THESIS

# NONCYTOPATHIC TYPE 2 BOVINE VIRAL DIARRHEA VIRUS 96B2222 INDUCES UPREGULATION OF TYPE-I INTERFERON AND CHEMOKINE RECEPTOR 4 EXPRESSION IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS DURING IN VITRO INFECTION

Submitted by

Cristina M. Weiner

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2010

COLORADO STATE UNIVERSITY

April 1, 2010

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY CRISTINA M. WEINER ENTITLED NONCYTOPATHIC BOVINE VIRAL DIARRHEA VIRUS 96B2222 INDUCES UPREGULATION OF TYPE-I INTERFERON AND CHEMOKINE RECEPTOR 4 EXPRESSION IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS DURING INFECTION IN VITRO BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate work

Hana Van Campen

Advisor: Sue Vandewoude

Co-Advisor: Thomas R. Hansen

Department Head: Edward Hoover

ABSTRACT OF THESIS

NONCYTOPATHIC TYPE 2 BOVINE VIRAL DIARRHEA VIRUS 96B2222 INDUCES UPREGULATION OF TYPE-I INTERFERON AND CHEMOKINE RECEPTOR 4 EXPRESSION IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS DURING INFECTION IN VITRO

Bovine viral diarrhea virus (BVDV) infection leads to monetary losses in cattle operations due to morbidity and impaired growth. IT is not currently possible to identify pregnant cattle carrying fetuses persistently infected (PI) with BVDV. We previously described a type-I interferon (IFN-I) response to acute non-cytopathic (ncp) BVDV infection in PI fetuses. It was hypothesized that infection of peripheral blood mononuclear cells (PBMC) with ncp-2 96b2222 BVDV would: (1) up-regulate IFN-stimulated genes (*IFN-β*, *ISG15),* antiviral molecules (retinoic acid inducible gene I, *RIG-I*) and chemokine receptor four (*CXCR4*); (2) viral entry would be mediated through CXCR4; and (3) differentially affect immune cell populations and expression of CXCR4. After optimization of *in vitro* conditions, PBMCs isolated from a BVDV-naïve steer were infected with ncp-2 96b2222 BVDV and treated with AMD3100 and/or CXCR4's ligand, chemokine ligand 12 (CXCL12). Cells were collected and processed for RT-PCR and flow cytometry staining post-infection. At 32 hours post-infection (hpi), mRNA expression of IFN-I pathway genes (*IFN-β, ISG-15*), antiviral molecules (*RIG-I*), *CXCR4, CXCL12* and *CD8* increased ($P < 0.05$). Treatment of PBMCs with AMD3100 prior to BVDV infection did not affect BVDV mRNA replication but caused a decrease in

CXCR4 mRNA and cell surface expression. CXCL12 treatment increased the concentration of *BVDV, CXCR4*, *IFN- β, ISG15*, and *RIG-I* transcription. Blocking CXCR4 with AMD3100 and/or CXCL12 did not prevent viral entry and/or replication, but abrogated a BVDV-induced increase in *CXCR4* mRNA expression while CXCL12 modulated infection. During PBMC infection *in vitro*, BVDV may induce release of IFN-β which then activates ISGs to increase *CXCR4* mRNA and protein. Flow cytometry data suggest trends in immune cell populations during ncp-2 96b2222 BVDV infection.

Keywords: Bovine viral diarrhea virus (BVDV), IFN-I, chemokine receptor four (CXCR4), flow cytometry

Cristina M. Weiner
Microbiology, Immunology, and Pathology
Colorado State University
Fort Collins, CO 80523
Spring 2010

## ACKNOWLEDGEMENTS

I would like to thank Dr. Thomas R. Hansen for allowing me to learn in his laboratory at the ARBL. I entered the combined DVM residency/MS degree program with a lot of enthusiasm for investigative research, and he has helped keep that interest alive in me. I would also like to thank Dr. Sue Vandewoude for her mentorship, advice, friendship and guidance during my Comparative Medicine residency and many projects through LAR and MIP. Thank you to Dr. Hana Van Campen for providing many useful suggestions and guidelines for the experiments, and to Dr. Natalia Smirnova who was instrumental in helping me to orient myself within the laboratory and providing input into and analysis of the experiments in this manuscript. A special thank you to Drs. Vandewoude and Van Campen for providing the Hansen laboratory with materials for the *in vitro* experiments: Dr. Vandewoude provided the AMD3100 and Dr. Van Campen provided the virus.

I would also like to thank the other Hansen and ARBL lab members for their assistance in answering the abundant questions I generated throughout my experiments; many thanks to Rick Brandes, Mike Gallegos, and others involved in the ARBL farm crew for their assistance in blood collections. Additionally, I would like to thank my comparative medicine resident-mates, Drs. Wilkerson, Rosenbaum, Magden, and Burgess for helping me tremendously along the way.

Of course, I would like to thank my family for supporting me during my residency and research projects. My parents have had constant inquiries about and curiosity for what I am learning. My brother Kevin has been an invaluable resource for perspective on all things scientific and somewhat arbitrary. Lastly and most importantly, thank you to my husband, Tyler, for his love and support during our adventure in Colorado. I could not have finished these projects without his steadfast encouragement, cooperation, and love.

# DEDICATION

For Tyler, my love for whom knows no bounds. For my twin brother Matthew, whose life and passing inspires me to walk along the path of the unknown and unexplained.

TABLE OF CONTENTS

# CHAPTER I: REVIEW OF THE LITERATURE

## 1. Introduction

Bovine Viral Diarrhea Virus (BVDV) and the subsequent disease it causes are responsible for ongoing animal loss and morbidity as well as economic deficits for cattle producers (Houe, 2003). The virus is exceptionally difficult to eliminate from a herd, and as such BVDV remains a problem for cattle populations in the United States and elsewhere. Understanding key components in virus's recognized history, methods the virus uses to evade the host immune response, and mechanisms by which the virus might interact with molecules during infection, may support a better understanding for how the virus has persevered. The mechanisms by which the virus evades the host immune response are not well understood. Previous studies in our laboratory have shown that persistently infected (PI) animals have an innate immune response against ncp-2 96b2222 BVDV, characterized by a type-I interferon response (IFN-I) (Shoemaker et al., 2008). We were interested in further investigating the role of the innate immune response, chemokines and their receptors in BVDV infection during *in vitro* infection to develop a more accessible model of PI.

## 2. Identifying the Etiologic Agent of the Disease

Bovine viral diarrhea virus has vexed the veterinary and cattle production communities since it was first described subsequent to disease outbreak in Saskatchewan

in the 1940s (Goens, 2002; Olafson P, 1946). At this time, the "X" disease was described as occurring as an acute or subacute syndrome with distinct clinical signs. The acute form persisted for seven to ten days in young animals and usually ended in death; mature cattle had different signs and died within 3-4 days after signs first appeared (Childs, 1946; Goens, 2002). Clinical signs included the onset of explosive, profuse watery and bloody diarrhea. The animals also became dehydrated, had an increased body temperature, would strain to defecate, and had increased heart and respiratory rates. Additionally, animals had drooping ears, anorexia, excessive lacrimation, nasal discharge, hypersalivation, and ulcer development at the nares, muzzle, lips and oral cavity (Childs, 1946; Goens, 2002; Olafson P, 1946). The subacute form was thought to linger in the environment for years and contribute to the sporadic death of 1 to 2 animals every several weeks. Clinical signs were similar to the acute form, only they were milder.

The veterinary and scientific communities were motivated to pursue an etiology for this mysterious and devastating disease. In 1946, Cornell University reported an outbreak of an undescribed transmissible disease in cattle that was similar to the reports from Western Canada (Goens, 2002; Olafson P, 1946). This new disease was similar to "X" disease, but animals affected with it had distinct clinical signs. This latter form was characterized by emaciation, thick and dry skin, proliferative lesions on the tongue, palate, and esophagus, and thickening of other epithelial tissues (Goens, 2002; Olafson P, 1946). Clinical signs not before noted in "X" disease, including respiratory distress, leucopenia, decreased milk production and a rise in abortion rates were noted (Goens, 2002; Olafson P, 1946). No bacteria were cultured from samples collected from affected

animals, and so the etiology was attributed to viral origins. Soon, "X" disease became known as Viral Diarrhea (VD) of cattle (Goens, 2002; Olafson P, 1946).

In the 1950s, a distinct form of VD was reported in Iowa. Nasal discharge was purulent, the disease usually did not recur on the same farm for years, and efforts to reproduce the disease in other animals were unrewarding (Goens, 2002; Underdahl et al., 1957). Because of the gross lesions associated with this different form, it became known as Mucosal Disease (MD) of cattle (Goens, 2002; Underdahl et al., 1957).

In 1957, researchers isolated a cytopathic virus from a case of MD (Goens, 2002; Underdahl et al., 1957). At Cornell University that same year, a noncytopathic virus was isolated from a case of VD (Goens, 2002; Lee and Gillespie, 1957). With much scientific diligence and by the end of the 1960s, the two disease forms were ultimately linked to one cause (Goens, 2002; Jubb and Kennedy, 1963). The complicated pathogenesis of what ultimately was termed bovine viral diarrhea-mucosal disease complex (BVD-MD) was studied for years, and ultimately it was concluded that neonatal calves were exposed to the virus during gestation, were persistently infected with the virus, and lacked circulating antibodies against the virus (Brownlie et al., 1984; Coria and McClurkin, 1978; Goens, 2002; Johnson and Muscoplat, 1973; McClurkin et al., 1984).

A great deal of specificity and detail evolved in science and the specialty of virology during the next several years. By the 1980s, BVDV was classified as a pestivirus (Radostits OM, 1988) in the family Flaviviridae, and it is a single-stranded positive sense RNA virus (Hamers et al., 2001; Kummerer et al., 2000). The virus lacks a proofreading mechanism and is therefore highly mutagenic, a characteristic through which the virus is

able to proliferate, propagate and wreak havoc on its host organism (Desport et al., 1998; Ridpath, 2003; Ridpath et al., 1994).

In the United States and elsewhere, the virus is characterized in 2 ways: biotype and genotype. Biotypes of BVDV include cytopathic (cp) and noncytopathic (ncp) forms. The difference between these is thought to be due to recombination of the viral genome and resulting in truncation of the nonstructural genes (NS) NS2/3 as well as gene products (Ridpath, 2003). Biotype classification is based on the viral strain *in vitro* and is not related to virulence (Aiello, 1998). Noncytopathic strains do not kill the cell, while cytopathic strains do. The second method of classification is by genotype. There are up to 12 different genotypes of BVDV, although classification in the United States is primarily restricted to types 1a, 1b, 2a, and 2b (Aiello, 1998). The differences in viral genotype are attributed to genetic drift within types 1 or 2, and the incorrect incorporation of nucleotides during genetic recombination (Aiello, 1998). Noncytopathic type 2 BVDV will be the focus of this literature review for the purposes of this manuscript.

### 3. Viral Transmission

BVDV is transmitted both horizontally and vertically. All animal excretions and secretions appear to be effective vehicles for spreading the virus between animals. The virus may also be spread via biting insects, fomites or wild ruminants (Aiello, 1998) as well as aerosol transmission (Uttenthal et al., 2006). Virus may also be spread through semen or vaginal discharge during artificial or natural breeding (Kirkland et al., 1991; Paton et al., 1989). Perhaps a more foreboding method of transmission is vertically from

dam to fetus. Viral exposure can result in various forms of fetal infection dependent on the viral biotype and stage of gestation.

Dams exposed to the BVDV virus via vaccination or previous infection prior to pregnancy develop a titer of neutralizing antibodies and infection of the fetus with the homologous BVDV strain does not occur. However, if infection with a ncp BVDV occurs after conception and prior to day 150 of gestation, then the virus invades the placentome and infects the fetus. The fetus subsequently becomes persistently infected (PI) because viral antigen is present prior to education of B and T lymphocytes and is recognized as "self" (McClurkin et al., 1984; Stokstad and Loken, 2002). This results in a fetal adaptive immunotolerance of viral antigen. However, if the dam is infected with a ncp BVDV after day 150 of gestation, the viral antigen is present after the immune education in the fetus; the fetus becomes transiently infected and ultimately may clear the virus and is able to mount an adaptive immune response (McClurkin et al., 1984; Shoemaker et al., 2009; Smirnova et al., 2008; Stokstad and Loken, 2002).

**4. Clinical Disease in Persistent Infection**

Although cp BVDV impairs cells *in vitro*, *in vivo* infection is not usually as striking. If an immunocompetent animal is infected with a cp BVDV, then infection results in the production of neutralizing antibodies against the virus, and the infection is cleared (Ohmann et al., 1987). Infection of the dam during early gestation can result in termination of the pregnancy due to abortion or embryonic loss. However, infection with a cp BVDV during late gestation usually does not adversely affect the developing fetus (Brownlie et al., 1989), as cp BVDV does not result in PI because it elicits a strong innate

immune response. Perhaps more significantly, cp BVDV leads to a strong IFN response in cattle at an early stage of gestation (Brownlie et al., 1998; Rinaldo et al., 1976). Thus, infection of healthy animal with a cp BVDV strain results in acute clinical disease and the effectual resolution of the virus.

There are a number of clinical ramifications and production effects that are associated with ncp BVDV PI, which are more dramatic compared to cp BVDV infections (Bielefeldt-Ohmann, 1995). These consequences range from adverse effects on growth and development, such as intrauterine growth restriction and malformations of long bones, to malformations of the central nervous system and generalized immune suppression as adults (Brownlie et al., 1998; Constable et al., 1993; Houe, 2003; Shoemaker et al., 2008). However, PI animals can also present clinically as relatively normal animals (Fredriksen et al., 1999; Ohmann, 1982; Swasdipan et al., 2002). This masquerading presentation allows PI animals to remain undetected in the herd and permits a constant, hidden source of viral propagation and transmission between animals (Confer et al., 2005; Fulton et al., 2005).

Persistent infection in calves also allows for the development of mucosal disease. This occurs when an animal PI with ncp BVDV is exposed to a homologous cp BVDV strain of the virus which may occur in one of two ways. In the first, the ncp strain mutates to form a cp strain. In this scenario, the animal is exposed to an antigenically similar version of the ncp virus (Brownlie et al., 1984; Goens, 2002; Sentsui et al., 2001). Because of this similarity, the animal is unable to mount an immune response against the dissimilar strain. The second occurs when a PI animal is exposed to a cp BVDV strain

through virus persisting in the environment, modified live vaccines, or other animals acutely infected with a cp BVDV strain. In these cases, the animal is usually able to mount an antiviral response against the different strain. Although development of mucosal disease is infrequent, it can be very severe and is invariably lethal in affected animals (Aiello, 1998).

**5. The Immune Response in Animals PI with ncp BVDV**

Fetal persistent infection represents the hallmark of BVDV perseverance within the herd. The classical description of fetal immune response during ncp BVDV intrauterine infection has focused on the innate immune response, which is triggered by recognition of components of common pathogens (Janeway CA Jr, 2005). Viral pathogens are recognized by RNA helicases, such as retinoic acid-inducible gene I (RIG-I), in the cytosol , which stimulates signal transduction cascades and leads to the production of IFN-I molecules, such as IFN α and IFN β (Wilkins and Gale). These IFN-I molecules are released and recognized by IFN receptors, which activate signal transduction cascades, such as the JAK/STAT pathway, leading to the activation of interferon stimulated genes (ISGs) (Wilkins and Gale). This activation pathway also promotes an antiviral state within the host cell, and in an immunocompetent animal, can initiate the adaptive immune response (Janeway CA Jr, 2005). In a number of different *in vitro* experiments, other groups have postulated that the virus is able to evade the innate immune response via manipulations of the IFN-I pathway (Baigent et al., 2002; Charleston et al., 2001; Chen et al., 2007; Gil et al., 2006a; Gil et al., 2006b; Schweizer et al., 2006; Schweizer and Peterhans, 2001). However, recent studies have shown ncp-2

BVDV infection induces an innate immune response via IFN-I molecule expression in PI *in vivo* tissues and during PI *in vitro* (Brackenbury et al., 2005).

Data from our laboratory has corroborated the literature that shows that maternal infection at day 175 of gestation with a ncp BVDV results in transient infection of the fetus (Goyal, 2005). With this strain of virus, the fetal immune response is characterized by a strong interferon response and a low viral RNA (Shoemaker et al., 2008; Smirnova et al., 2008). However, maternal infection at day 75 of gestation causes persistent infection of the fetus and a high viral RNA load and a mild interferon response (Shoemaker et al., 2008).

Blood was collected from heifers carrying PI fetuses at day 160 gestation for microarray analysis to investigate differences in animals carrying infected or uninfected offspring. Results indicated a down-regulation of novel molecule to the BVDV arena, chemokine receptor 4 (CXCR4) at day 160 of gestation (Smirnova et al., 2008). Further evaluation of this tissue indicated that the down-regulation of CXCR4 was evident for approximately three months in these animals (Smirnova et al., 2009). In contrast, there was an up-regulation of CXCR4 expression in whole blood in PI fetal animals collected at day 190 gestation (Shoemaker ML, unpublished data 2008).

Given these observations, we investigated the connection between IFN-I, CXCR4, and BVDV during ncp-2 BVDV infection; such a connection had not been made before (Smirnova et al., 2009). Interestingly, CXCR4 expression has been shown to be induced by IFN-I during other viral infections (Serra et al., 2008), and alterations in CXCR4 expression or function as a result of ncp-2 BVDV infection might have implications on

immune response against the virus. CXCR4 and its natural ligand, chemokine lingand 12 (CXCL12), have been shown to regulate fetal development in other species. Therefore, aberrations in this CXCR4:CXCL12 axis in the developing fetus exposed to ncp-2 BVDV might have an impact on fetal development and development of PI.

## 6. Chemokines, Chemokine Receptors and Their Regulation

Chemokines are 8-10 kD molecules that influence leukocyte chemotaxis. Chemokines are classified into four different groups based on the location of the first one or two cysteine residues (Busillo and Benovic, 2007; Volin et al., 1998). They are G-protein-coupled receptors and couple to pertussis-sensitive $G_i$ proteins, and in general the chemokine-chemokine receptor relationships are promiscuous (Busillo and Benovic, 2007). However, CXCR4 and its natural ligand CXCL12 are considered monogamous (Amara et al., 1997; Busillo and Benovic, 2007; Jones and Nelson, 2007; Kumar et al., 2006; Maekawa and Ishii, 2000; Sodhi et al., 2004; Zou et al., 1998), although CXCL12 has recently been shown to bind receptors other than CXCR4 (Hatse et al., 2006). CXCR4 is a 352 amino-acid rhodopsin-like G-protein-coupled receptor (GPCR), and it is highly expressed in the thymus, particularly in immature CD4+ and CD8+ cells. During early lymphocyte development, there are few mature thymocytes, as emigration does not occur until after birth through a process that involves $G_i$ proteins (Zou et al., 1998). CXCL12 is constitutively secreted by marrow stromal cells (Burger and Burkle, 2007; Burger and Peled, 2009). Further, chemokines can act during inflammation to attract inflammatory cells or during homeostasis to coordinate cell trafficking (Burger and Peled, 2009).

CXCR4 expression is regulated at a number of levels. It has been shown that Nuclear Respiratory Factor-1 (NRF-1) is the main transcription factor that positively regulates transcription (Moriuchi et al., 1997; Wegner et al., 1998), although it has also been suggested that stimulatory protein 1 (SP-1) may also positively regulate transcription (Wegner et al., 1998). Ying-Yang-1 (YY-1) has been shown to negatively regulate transcription (Moriuchi et al., 1999). At the transcriptional level, expression is increased by intracellular calcium, cyclic AMP, interleukins 2, 4, 7,10, 15; TGF-$\beta$, simultaneous CD3 and CD28 engagement, bFGF, VEGF, and EGF. Expression is attenuated by TNF-$\alpha$, IFN-$\gamma$, and IL-1$\beta$ (Busillo and Benovic, 2007). Protein expression is thought to be regulated both by glycosylation and tyrosine sulfation, and alterations to the latter can affect ligand binding (Busillo and Benovic, 2007).

Additionally, regulation can occur at the level of oligomerization. CXCR4 is heterogeneous in cells, but can both homodimerize and heterodimerize (Busillo and Benovic, 2007). In the absence of CXCL12, it has been suggested that CXCR4 homodimerizes; other reports suggests that CXCL12 enhances CXCR4 dimerization (Busillo and Benovic, 2007).

CXCR4 signaling via CXCL12 binding is thought to be regulated in two steps. First, a conformational change occurs when CXCL12 interacts with CXCR4, allowing complete binding (Crump et al., 1997). Inflammation positively and negatively regulates the CXCL12/CXCR4 axis. In an inflammatory reaction, neutrophils release cathespin G and neutrophil elastase, which inactivate CXCL12 by cleaving the N-terminal residues needed to interact with CXCR4; additionally, neutrophil elastase cleaves the N-terminal

domain of CXCR4 (Busillo and Benovic, 2007). CXCL12 has also been shown to interact with glycosaminoglycans (GAGs) and may enhance oligomerization, which elicits CXCL12- mediated inhibition of HIV in the presence of heparin sulfate (Busillo and Benovic, 2007).

CXCR4 signaling pathways are pertussis toxin (PTX) -sensitive and are dependent on $G_i$ proteins. However, the activation of JAK/STAT by CXCR4 is G-protein independent. CXCL12 has been shown to induce a transient association of JAK 2/3 with CXCR4, resulting in a translocation of many STAT proteins. Pre-treating cells *in vitro* with PTX led to a prolonged association of JAK with CXCR4, and so it is concluded that G-protein coupling is involved in JAK/STAT receptor complex recycling. Further, CXCR4 can be desensitized, internalized, and degraded (Busillo and Benovic, 2007).

Kumar et. al. showed that CXCR4 heterodimerizes with the T-cell-receptor (TCR) to signal in T-cells via CXCL12. CXCR4 utilizes the ZAP70 ITAM binding domains for TCR signal transduction (Kumar et al., 2006). ZAP70 expression is required for CXCL12 to stimulate prolonged ERK activation as well as to stimulate a robust Ras activation (Kumar et al., 2006). Kumar et. al. also showed that treatment of T cells with CXCL12 activated multiple downstream signaling pathways, prolonging activation of the extracellular signal-regulated kinases (ERK-1, 2). CXCL12-mediated ERK-pathway activation induces multiple genes that regulate T-cell signal transduction, adhesion, cell shape, DNA repair, and apoptosis (Kumar et al., 2006). These studies suggest that CXCR4 signaling utilizes pre-existing, constitutively-expressed tyrosine phosphorylated TCR-ZAP70 complexes.

## 7. CXCR4 and its Role in Development

CXCR4 has been implicated in a number of developmental processes, including hematopoiesis, angiogenesis, and organogenesis (Busillo and Benovic, 2007; Maekawa and Ishii, 2000; Zou et al., 1998). This process has been described in the mouse, although explicit knowledge of the homing process is lacking (Maekawa and Ishii, 2000). Murine hematopoiesis begins in the yolk sac at nine days-post-coitus (dpc), moves to the paraaortic-splanchnopleura/aortic-gonad-mesonephros region by 10 dpc, and then migrates through the liver to reach the bone marrow by 16 dpc. Ultimately, these cells move on to the thymus and other lymphoid tissues after birth (Maekawa and Ishii, 2000).

Genetically modified mice deficient in CXCL12 exhibit specific phenotypes: half died between 16.5 and 18.5 dpc, while the remainder survived to birth, but died within hours (Maekawa and Ishii, 2000; Zou et al., 1998). CXCL12-deficient animals had fewer B cell progenitors than wild-type animals, and impairment of lymphoid and myeloid progenitors in bone marrow is present (Maekawa and Ishii, 2000; Zou et al., 1998). Discrepancy in the hematopoietic process between the liver and bone marrow in CXCL12-deficient animals suggests a role for homing of these progenitor cells from the liver to the bone marrow (Maekawa and Ishii, 2000).

CXCR4-deficient mice do not survive to parturition. In the fetal liver of CXCR4-deficient animals, the number of B-cell progenitors is reduced, while the number of myeloid progenitors is normal in the liver, but they are reduced in the bone marrow (Maekawa and Ishii, 2000; Zou et al., 1998). Again, this suggests a role for CXCR4 expression in the trafficking of cells from the liver to the bone marrow (Maekawa and

Ishii, 2000). CXCR4-deficient animals have an increased incidence of ventricular septal defects, anomalies of the great vessels feeding the gastrointestinal tract, and abnormal granule cell layers in the cerebellum (Maekawa and Ishii, 2000).

Both CXCR4 and CXCL12 have been shown to be important for trafficking hematopoietic cells from the liver to the bone marrow, and mouse models that are deficient in these molecules have lesions including cardiac defects, vascularization defects, and defects in the CNS. These defects have also been noted in calves PI with ncp-2 BVDV. We were therefore interested to evaluate whether aberrations in CXCR4 and/or CXCL12 expression might be present during ncp-2 BVDV infection that might lend insight into the lesion development in PI animals.

**8. CXCR4 and Viral Infection**

CXCR4 has been implicated in a wide range of virally-induced diseases and studied extensively in HIV infection because of its role as an entry receptor (Maekawa and Ishii, 2000; Sodhi et al., 2004; Volin et al., 1998). BVDV viral entry has been shown to occur via clathrin-mediated endocytosis (Lecot et al., 2005) as well as dependence on heterodimer formation of the viral envelope proteins E1/E2 (Ronecker et al., 2008). . Blocking CXCR4 with small ligands, such as AMD3100, effectively interferes with HIV entry (Zou et al., 1998).

Down-regulation of CXCR may be an antiviral principle (Amara et al., 1997). The mechanism by which AMD3100 binds to CXCR4 is well-described; it has been shown to initiate a rapid and transient leukocytosis due to mobilization of hematopoietic cells into the blood from the bone marrow *in vivo* (Burger and Peled, 2009). Unlike

binding CXCR4 with AMD3100, the interaction with CXCL12 is functional; CXCL12 is targeted to the endoplasmic reticulum to inactivate CXCR4 transcription, which results in down-regulation of CXCR4 expression (Zhang et al., 2009). Others have shown that recombinant manipulation of CXCL12 can lead to a decrease in viral entry via a down-regulation in CXCR4 (Zhang et al., 2009).

## 9. Conclusion and Hypotheses

Ultimately, the immune response against BVDV as it relates to CXCR4 expression has not been investigated. Our laboratory has noted an increase in CXCR4 expression in PI fetal blood (MS Shoemaker thesis, unpublished data), while Smirnova et. al. found CXCR4 expression down-regulated in animals carrying PI fetuses (Smirnova et al., 2009). These maternal data are contrary to the PI fetal data, which might suggest that the maternal change is a response to what is occurring in the fetus during its development in the presence of virus. To model the PI immune response and investigate CXCR4 expression, we developed an *in vitro* model for BVDV infection with bovine peripheral blood mononuclear cells (PBMCs), as PBMCs sustained in cell culture and infected *in vitro* should have the capacity to produce IFN-I and express CXCR4. These features are also noted to occur in PI animals. We hypothesized that infection of PBMCs with ncp-2 96b2222 BVDV *in vitro* will mount an IFN-I response and be mediated through and increase CXCR4 and CXCL12.

## CHAPTER II: METHODS FOR OPTIMIZATION OF IN VITRO CONDITIONS

### 1. Introduction

We hypothesized that infection of PBMCs with ncp-2 96b2222 BVDV *in vitro* will mount an IFN-I response and be mediated through and increase CXCR4 and CXCL12. Our laboratory was interested in optimizing *in vitro* conditions to model natural acute infection of ncp-2 BVDV. Cells of bovine origin were used since natural infection with the virus is specific to this species. Experiments were designed to evaluate: 1) the infective capacity of a commercially-available bovine-derived cell line (ATCC CRL-6017); 2) optimal conditions for incubating virus with PBMCs after adsorption time; 3) different multiplicity of infection (moi) in bovine PBMCs; 4) a pilot experiment evaluating CXCR4 and T-cell receptor (TCR) pathway gene expression on bovine PBMCs; and 5) a pilot experiment evaluating AMD3100 treatment concentrations in bovine PBMCs infected with ncp-2 96b2222 BVDV.

*1.1 Cell line ATCC #6017*

A uniform cell population that would become consistently and homogenously infected with ncp-2 BVDV was sought. This would create optimized *in vitro* conditions to better evaluate the pathogenesis and cell signaling cascades associated with acute infection *in vitro*. Successful development of an *in vitro* model of reliable acute infection would allow for the investigation *in vitro* of acute and persistent infection. An *in vitro*

model was preferable in order to minimize factors associated with housing an animal for the purposes of repeated PBMC isolation, stress from repeated blood collections, cost associated with long-term housing of this animal, and assurance of use of a BVDV-free source. Although there are few commercially-available bovine-derived cell lines, a supply of bovine mononuclear cells was harvested from bovine lymph nodes in Dr. John Dame's laboratory at the University of Florida. This line has an ATCC number CRL-6017. Although this cell line has an ATCC number, it is part of the Naval Biosciences Laboratory (NBL) collection. As a result, ATCC does not have accession information, nor does it guarantee that the morphology or activity of this cell line remain consistent. Despite this, these cells were used for the cell line experiments since it seemed to be an appropriate source and cell population for BVDV infection.

*1.2 Wash/No wash pilot experiment*

The *in vitro* effects on PBMCs were evaluated when the virus was present for the duration of the infection. An experiment that would compare viral mRNA expression was designed between PBMCs exposed to virus for a limited period of time (60 minutes) to allow for viral adsorption and PBMCs exposed to virus for the duration of the experiment (72 hours). Then these cells were evaluated for viral and *CXCR4* mRNA expression.

*1.3 Multiplicity of infection experiment*

As stated previously, ncp-2 BVDV can infect and be isolated from many different tissue types, and different quantities of virus may be harvested from different animals.

Different multiplicities of infection were evaluated to optimize the infection conditions *in vitro* as to whether changes would alter the infection rates of PBMCs.

*1.4 CXCR4 and T-cell Receptor pathway genes pilot experiment*

A pilot experiment was conducted to evaluate whether CXCR4 and other TCR pathway gene expression changed during acute infection *in vitro*, as these targets were identified previously with microarray analysis to change during the course of acute and resolving maternal infection (Smirnova et al., 2009).

*1.5 Blocking experiments—pilot study*

The effectiveness of AMD3100 for blocking CXCR4 and viral cellular entry was examined, but the appropriate concentrations or infection durations were not clear. During other viral infections, namely HIV and FIV, various ranges of AMD3100 concentrations are used in dose-dependent inhibition studies (Smirnova et al., 2005). However, the number of cells available was limited since PBMCs harvested and isolated were from single blood collections. Thus, a pilot experiment was designed to evaluate whether treatment with two representative AMD3100 concentrations would differentially affect BVDV RNA expression.

**2. Materials and Methods**

*2.1 ATCC #6017 Infection with ncp-2 96b2222 BVDV in vitro*

Cells were suspended in culture media RPMI-1640 supplemented with 1% Penicillin/Streptomycin/Amphotericin B and 10% certified BVDV-free fetal bovine serum (FBS) and were passaged in 75mm$^2$ flasks and incubated at 37°C and 5% $CO_2$.

Cells were adherent to the flasks and were harvested by incubating with trypsin for 10 minutes. Collected cells, suspended in culture media, were counted and plated in duplicate or triplicate into 6-well plates in 2 mL cell suspension (5 X $10^5$, 1 x $10^6$, 1.5 x $10^6$, or 2 x $10^6$ cells/well) for incubation experiments. Cells were incubated overnight at 37°C and 5% $CO_2$. Twenty four hours later, cells were infected with ncp-2 96b2222 BVDV at an moi of 1 and incubated with the virus for 60 minutes. Supernatant was removed and replaced with fresh media. Cells were sampled at multiple interval hours post-infection (hpi), as described below. Cell viability was assessed at each collection time point. Samples were collected for RT-PCR; targets were *GAPDH, TATA, CYCLOPHILIN, B-ACTIN, BVDV, IFN-β*, and *CXCR4,* and mRNA transcripts were quantified. The extended panel of housekeeping genes was necessary since the expression of relevant housekeeping genes was unknown for this cell population.

*2.2 Wash/No wash pilot experiment*

Forty milliliters (mL) whole blood was collected from a BVDV-naïve steer via the jugular vein into sodium-EDTA-coated collection tubes. All experiments with animals were approved by CSU's Institutional Animal Care and Use Committee. PBMC were isolated using Histopaque 1077 (Sigma-Aldrich, USA) following standard laboratory protocols. Whole blood was collected into a heparinized vacutainer, and RPMI-1640 media with Glutamax +25mM HEPES (Invitrogen Cat # 72400-054) and 1% penicillin/streptomycin & 1% fungizone was added 1:1. The blood/media mix was underlaid with Histopaque 1077, and cells were centrifuged at 400g for 30 minutes at room temperature. The buffy coat was removed, and the cells were washed twice in

RPMI-1640 media and centrifuged at 300g for 10 minutes at room temperature. Next, the cells were incubated for five minutes in a red blood cell lysis buffer and then washed again in RPMI-1640 media and centrifuged at 300 g for 10 minutes at room temperature. Cells were counted and 2 x $10^6$ cells/mL were suspended in RPMI-1640 (1%Penicillin/ Streptomycin/Amphotericin B) and 10% certified BVDV-free fetal bovine serum (FBS; Foundation Fetal Bovine Serum, cat #900-108, Gemini Bioproducts, Sacramento, CA USA). Cells were plated in triplicate into 12-well plates with 1 mL cell suspension (2 x $10^6$ cells/well) or 24-well plates with 0.25 mL cell suspension (5 x $10^5$ cells/well) for incubation experiments. After overnight incubation at 37°C and 5% $CO_2$ cells were either: 1) infected with moi 1 ncp-2 96b2222 BVDV and incubated with the virus for 60 minutes; then plates were centrifuged, supernatant was removed, and it was replaced with 2mL fresh media, referred to as the "wash" set-up or 2) infected with moi 1 ncp-2 96b2222 BVDV and incubated with the virus for the remainder of the experiment, referred to as the "no wash" set-up. Cells were sampled during the infection time course. Cells were incubated through 72 hpi for the "wash" experimental design, and through 60 hpi for the "no wash" experimental design. Cell viability was assessed at each collection timepoint.

*2.3 Multiplicity of Infection Experiments*

Blood was collected from a BVDV-naïve steer via the jugular vein into sodium-EDTA-coated collection tubes. PBMC were isolated using Histopaque 1077 (For details, see 2.2). Cells were counted and 2 x $10^6$ cells/mL were suspended in RPMI-1640 (1%Penicillin/ Streptomycin/Amphotericin B) and 10% certified BVDV-free FBS (for

details, see 2.2). Cells were plated in triplicate in 12-well plates with 1 mL cell suspension (2 x $10^6$ cells/well) or 24-well plates with 0.25 mL cell suspension (5x $10^5$ cells/well) for incubation experiments. After 24 hour incubation cells were infected with moi 1, 2 or 4 ncp-2 96b2222 BVDV and incubated for 60 minutes. Then plates were centrifuged, removed supernatant was replaced with 2mL fresh media and sampled at either six, eight, or 12-hour intervals post-infection. An moi of 1, 2 or 4 was used. Cell viability was assessed at each collection timepoint.

*2.4 CXCR4 and TCR pathway genes pilot experiment*

Cells were collected, isolated, plated, incubated and infected as above with an moi 1. Cells were sampled during the time course of infection for RT-PCR. Primers and probes for *CXCR4*, *ZAP70, CD3* ζ, and *MEK1/2* gene expression were tested as described.

*2.5 Pilot blocking experiments*

PBMCs were incubated overnight as described above. Prior to virus infection, 1.25 or 5 μg/mL AMD3100 was added to each well and incubated with cells for 15 minutes (Smirnova et al., 2005). Then, virus was added to the wells at moi 1 and cultured as described previously for 1 hr at 37°C and 5% $CO_2$. Cells were washed and media containing a final concentration of 1.25 or 5 μg/mL AMD3100 was added to each well for the remainder of the experiment. Cells were sampled at intervals for 48 h pi for RT-PCR for the target genes: *BVDV* and *CXCR4*. *GAPDH* was used as a housekeeping control gene.

*2.6 RT-PCR*

Cell samples were collected into TRI-reagent (Sigma, USA). RNA was isolated per manufacturer's instructions using the RNeasy MinElute cleanup kit (Qiagen, Valencia CA USA) and quantified using the NanoDrop spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA). The iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) constructed cDNA. *GAPDH, TATA, CYCLOPHILIN, B ACTIN, BVDV, IFN-B, CXCR4, ZAP70, CD3 Zeta,* and *MEK 1/2* were target genes for RT-PCR. Amplification was conducted on the LightCycler480 (Roche, Basal, Switzerland) with iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). Each reaction was carried out in duplicate and contained 2$\mu$L of cDNA template, 1.5 $\mu$L of a primer master mix containing 10$\mu$L each of 100uM forward and reverse primers diluted 1:13 in RNAse free water, 5$\mu$L of Supermix, and 1.5 $\mu$L RNAse-free water. The PCR reaction was carried out using the following protocol: 30s denaturation at 95°C, 30s annealing at 65°C, and 15s elongation at 70°C for 40 cycles. Fluorescence data were collected during all 40 cycles. PCR products were evaluated by gradual decreasing of temperature from 95°C to 55°C and collecting melt curve data for 8 minutes. Ct values were obtained from the Lightcycler software and analyzed in reference to *GAPDH* Ct values for each sample to determine $\Delta$Ct and relative expression of targets. Data were quantified as $2^{-\Delta Ct}$, and infected cell values were expressed as a fold change compared to control values, where applicable. Primer sequences are listed in Table 1.

The RT-PCR data were analyzed, and $\Delta$Ct values were generated. Statistical significance was determined using the Student's t-test.

**Table 1. RT-PCR primer sequences.**

| Target Gene | Primer Sequence |
|---|---|
| GAPDH | F:TGACCCCTTCATTGACCTTC<br>R:CGTTCTCTGCCTTGACTGTG |
| BVDV | F: TCGTCAATGGTTCGACACTC<br>R: CCGCATGGGTTAAGATGTG |
| CXCR4 | F:AAGGCTCAGAAGCGCAAG<br>R:GAGTCGATGCTGATCCCAAT |
| CXCL12 | F: CCTTGCCGATTCTTTGAGAG<br>R: GGTCAATGCACACTTGCCTA |
| IFN β | F: GATGCCTGAGGAGATGAAGA<br>R: GGTGAGAATGCCGAAGATGT |
| ISG 15 | F: GGTATCCGAGCTGAAGCAGTT<br>R: CCTCCCTGCTGTCAAGGT |
| RIG I | F: ACGTGCCAGAACAAATCAGA<br>R:TCTGGTTGAACCCTGACTGA |
| CD3 ζ | F:F:ACATAGCGGTGTCATTGCAG<br>R:CTCATTCCATGAGGTGAGCA |
| MEK 1/2 | F:GCATGCTTTGCTGCTATAAAAA<br>R: AAGGGCTCTGGCTAGATTTTG |
| ZAP70 | F: CTGCAAAGATTGACAGCAG<br>R:AGTGGCTGCACAAAATGACA |
| CD4 | F: GATCGAGGTCTTGCCTTCAG<br>R: GCAGCAAACAGTGAAAAGCA |
| CD8 | F:TGAGCAACCTGACCTCTGAA<br>R:GGTGGCCTCTCCTCTTTCAT |
| CD14 | F: CTCAGCGTGCTTGATCTCAG<br>R: AAGGGATTTCCGTCCAGAGT |
| MHC I | F: AGGACATGGAGCTTGTGGAG<br>R: TGGCACGTGTATCTCTGCTC |
| MHC II | F: CCTCGGATGATGAGGACAGT<br>R: TTGAGATTCCAGCCCCTATG |

Statistical significance was determined to be * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

## 3. Results

### *3.1 Cell line ATCC # 6017 Experiments*

The housekeeping genes *GAPDH, TATA,* and *B-ACTIN* did not reliably amplify within 40 cycles of RT-PCR, but the *CYCLOPHILIN* gene did. However, the *BVDV* mRNA was barely detected (Figure 2.1 a). CXCR4 mRNA expression was present in these cells as evidenced by increased mRNA concentration (Figure 2.1 b).

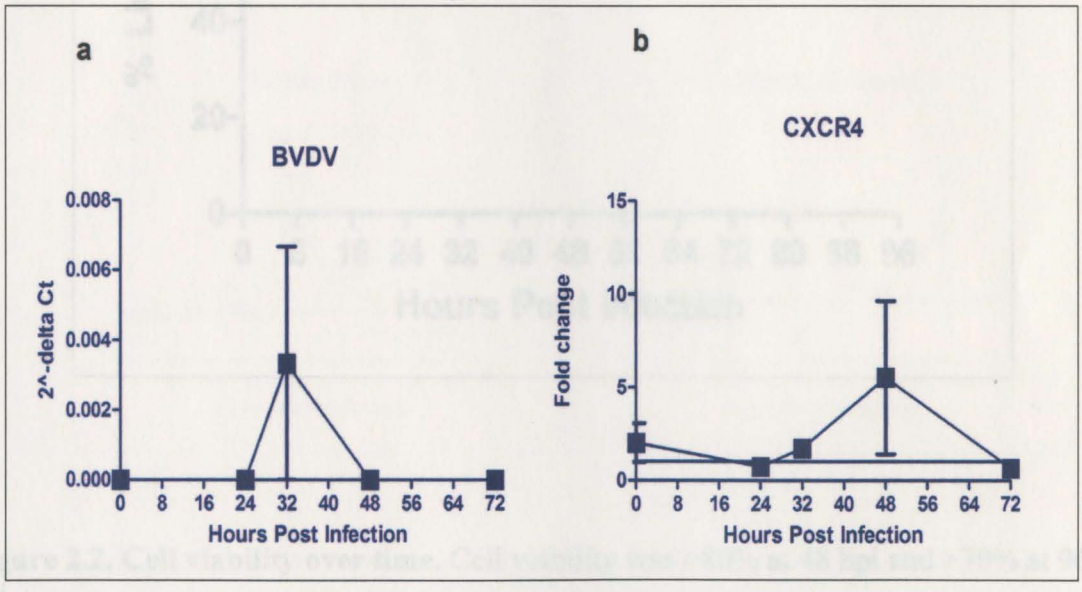


**Figure 2.1. RT-PCR mRNA expression.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ a) *BVDV* mRNA expression expressed as $2^{-\Delta Ct}$. There were no statistical differences. Viral mRNA concentration was minimal. b) *CXCR4* RNA expression as fold change with respect to control. RNA concentrations are expressed with respect to control concentrations. There were no statistical differences.

*3.2 Wash/No wash pilot experiment*

Cell viability was assessed at each time point during the experiment (Figure 2.2).

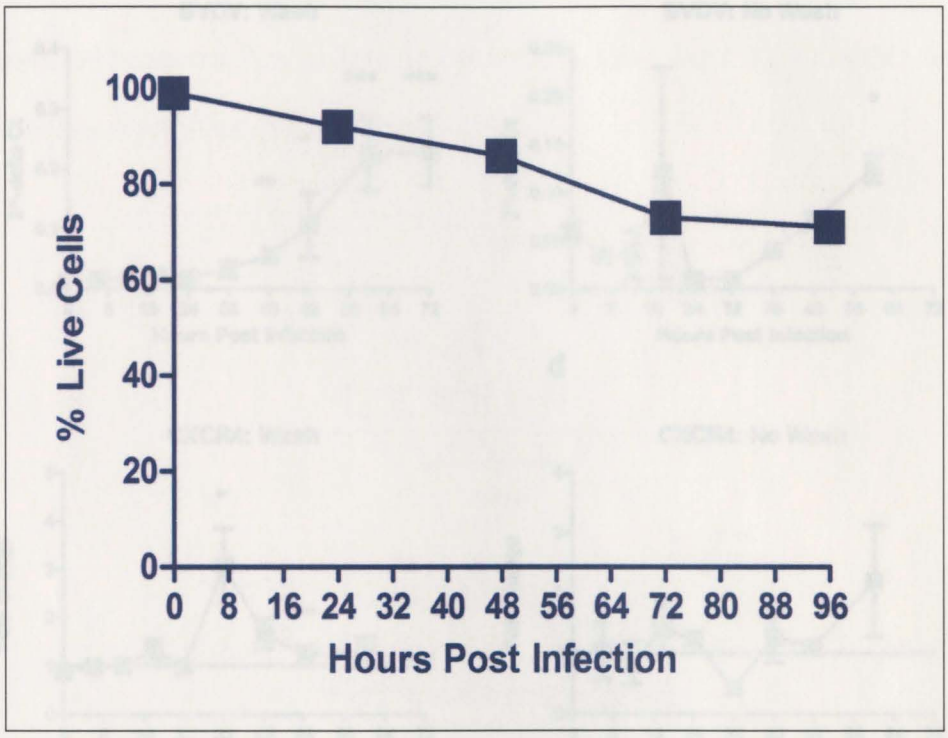


**Figure 2.2. Cell viability over time.** Cell viability was >80% at 48 hpi and >70% at 96 hpi.

*BVDV* mRNA results were more characteristic of viral growth and replication in culture over time in the "wash" experimental design. (Figure 2.3 a-d). The viral mRNA

concentration results were irregular in the "no wash" experimental design. For the remainder of all in vitro experiments, the "wash" experimental design was used.

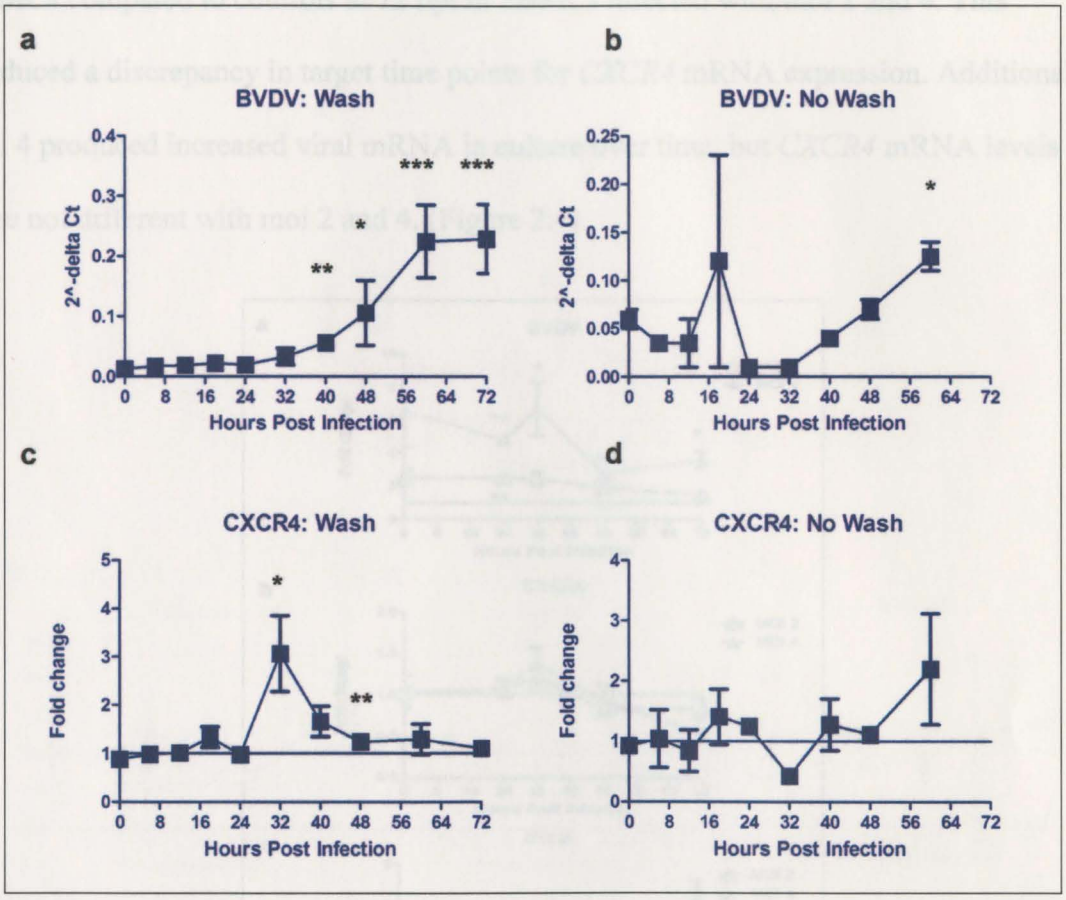


**Figure 2.3 RT-PCR mRNA expression in infected PBMCs for "wash" and "no wash" experimental designs.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ Concentrations are expressed as a fold change with respect to control for c-d. a) *BVDV* mRNA expression, "wash" experimental design. There was a significant difference between 0-40hpi ($P < 0.01$), 0-48 hpi ($P < 0.05$), and between 0- 60, 72 hpi ($P < 0.001$). b) *CXCR4* mRNA expression, "wash" experimental design. There was a significant difference between control and virus-treated PBMCs at 32 ($P < 0.05$) and 48 hpi ($P < 0.01$). c) *BVDV* mRNA expression, "no wash" experimental design. There was no statistical difference; however there was a higher mRNA concentration without removing the virus. d) *CXCR4* mRNA expression, "no wash" experimental design.

*3.3 Multiplicity of Infection*

There was an increase in *CXCR4* mRNA expression at 32 and 48 hpi in the previous experiment. However, we noted an increase in *IFN-β* mRNA concentration in infected PBMCs compared to controls at 72 hpi in PBMCs infected with moi 2 and 4. This produced a discrepancy in target time points for *CXCR4* mRNA expression. Additionally, moi 4 produced increased viral mRNA in culture over time, but *CXCR4* mRNA levels were not different with moi 2 and 4. (Figure 2.4).

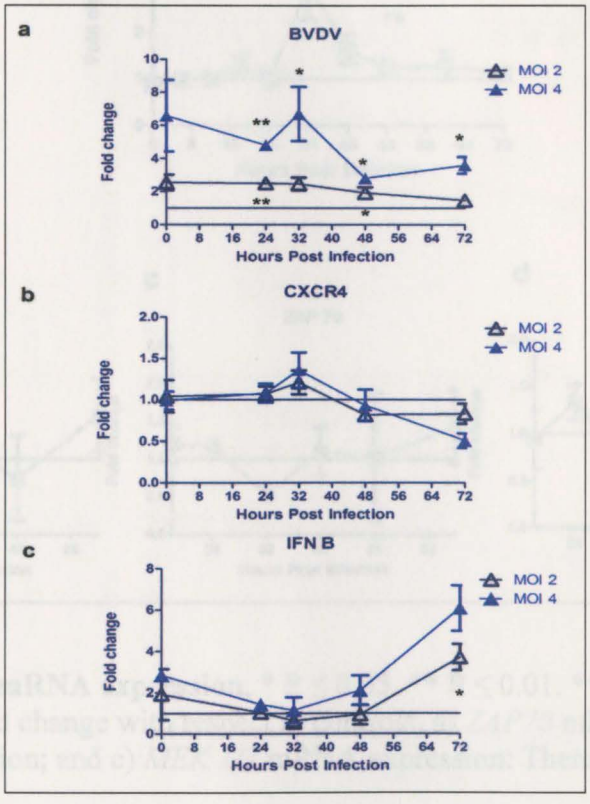


**Figure 2.4 RT-PCR mRNA expression, Multiplicity of Infection Experiment.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ Values are expressed as a fold change with respect to moi 1. a) *BVDV* mRNA expression. There were statistical differences between moi 1 and moi 2 and 4 at multiple timepoints. b) *CXCR4* mRNA expression. There were no statistical differences between mois. c) *IFN β* mRNA expression. There was a statistical difference between 60-72 hpi for moi 2 ($P < 0.05$) and moi 4 ($P < 0.01$).

*3.4 CXCR4 and TCR pathway genes pilot experiment*

*BVDV* mRNA expression increased over time in culture. *CXCR4* mRNA expression was increased in infected PBMCs compared to controls at 32, 40, and 48 hpi (Figure 2.5 a). Data for ZAP 70, CD3 ζ, and MEK 1/2 were unremarkable (Figure 2.5 b-d).

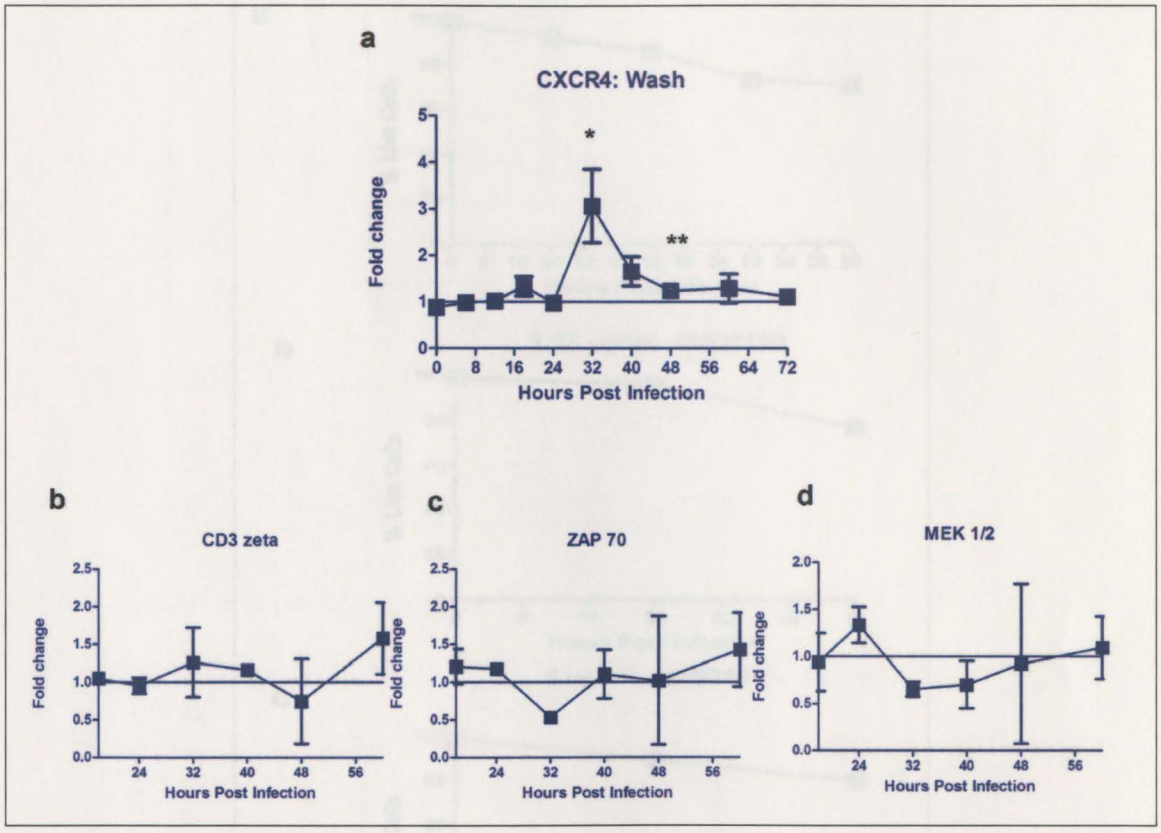


**Figure 2.5 RT-PCR mRNA expression,** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ Values are expressed as a fold change with respect to controls. a) *ZAP70* mRNA expression; b) *CD3ζ* mRNA expression; and c) *MEK 1/2* mRNA expression: There were no statistical differences.

*3.5 Pilot blocking experiments*

Cell viability was recorded at all time points during the infection to assess whether AMD3100 treatment at these concentrations was detrimental to bovine PBMCs (Figure

2.6). AMD3100 treatments were not successful in blocking the expression of *BVDV* mRNA. Infected PBMCs treated with the highest concentration of AMD3100 expressed the highest concentration of viral mRNA, while the lower AMD3100 concentration had the lowest viral mRNA concentration at 48 hpi (Figure 2.7a).

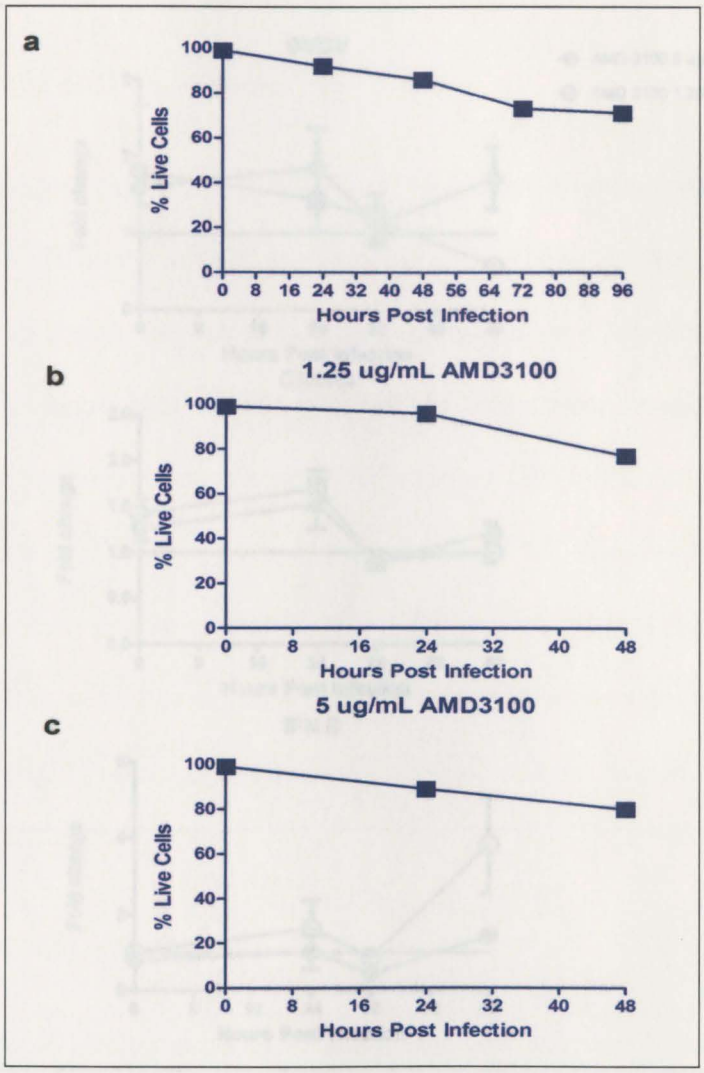


**Figure 2.6. PBMC viability during *in vitro* infection in the presence of AMD3100.** Viability remained >80% for 48 hpi. a) untreated cells. b) PBMCs treated with 1.25 μg/mL AMD3100. c) PBMCs treated with 5 μg/mL AMD3100.

Uninfected PBMCs treated with AMD3100 had higher *CXCR4* expression than untreated PBMCs (Figure 2.7 b). Treatment with the higher concentration of AMD3100 led to a slightly lower *CXCR4* mRNA concentration than the lower AMD3100 concentration at 24 hpi, but higher levels at 32 and 48 hpi. AMD3100 caused decrease of *CXCR4* mRNA

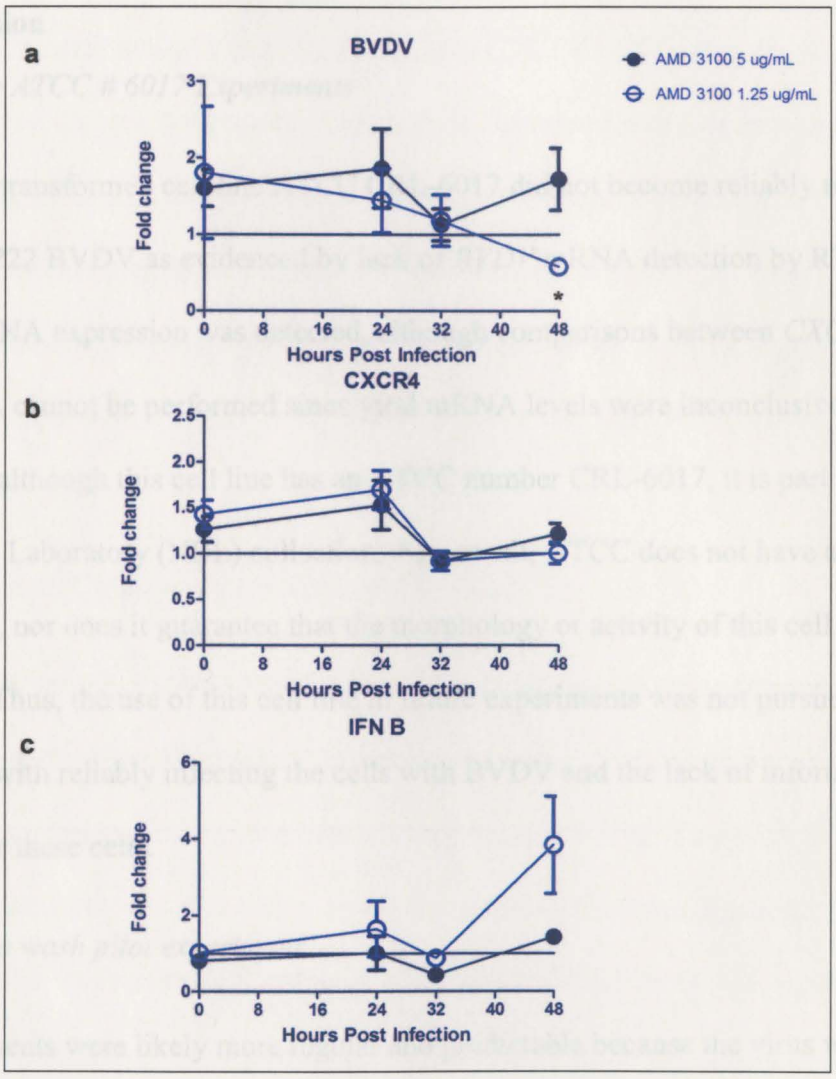


**Figure 2.7 RT-PCR mRNA expression.** Pilot AMD3100 Blocking experiment. Results are expressed as a fold change with respect to untreated infected PBMCs. * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ a) *BVDV* mRNA expression. There was a statistical difference between infected PBMCs and infected PBMCs treated with 1.25 µg/mL AMD3100 at 48 hpi ($P < 0.05$). b) *CXCR4* mRNA expression. There were no differences in CXCR4 expression. c) *IFN β* mRNA expression. There were no differences in *IFN β* expression.

expression in infected PBMCs at 32 hpi. *IFN β* mRNA expression increased in infected PBMCs (Figure 2.7 c). In the presence of both the higher and lower AMD3100 concentrations, *IFN β* mRNA expression appeared to be increased compared to infected PBMCs without treatment at 48 hpi.

## 4. Discussion

### *4.1 Cell line ATCC # 6017 Experiments*

The transformed cell line ATCC CRL-6017 did not become reliably infected with ncp-2 96b2222 BVDV as evidenced by lack of *BVDV* mRNA detection by RT-PCR. *CXCR4* mRNA expression was detected, although comparisons between *CXCR4* and viral mRNA cannot be performed since viral mRNA levels were inconclusive. As stated previously, although this cell line has an ATCC number CRL-6017, it is part of the Naval Biosciences Laboratory (NBL) collection. As a result, ATCC does not have accession information, nor does it guarantee that the morphology or activity of this cell line remain consistent. Thus, the use of this cell line in future experiments was not pursued due to the difficulties with reliably infecting the cells with BVDV and the lack of information available for these cells.

### *4.2 Wash/No wash pilot experiment*

Experiments were likely more regular and predictable because the virus was adsorbed with the PBMCs for a predetermined period of time and then was removed; any virus that had successfully infected the cells then had time to replicate and produce more viral mRNA during the length of the experiment. This appeared to be a more consistent

mechanism to measure viral mRNA concentration over time. The remaining in vitro experiments were conducted in this "wash" experimental design.

*4.3 Multiplicity of Infection*

Based on the results of the pilot study, an moi 1 was used for the subsequent experiments. At moi two and four, the increase in *CXCR4* mRNA expression at 32 hpi detected previously was not present. Although the increased moi was successful in producing higher concentrations of viral mRNA, it abrogated the pattern in *CXCR4* expression produced in earlier experiments and were interested in pursuing further. Reasons for the change remain unclear, but they might be the result of changes in CXCR4 signaling during higher viral loads. An moi 1 was used for all future *in vitro* experiments.

*4.4 CXCR4 and TCR pathway genes pilot experiment*

The *BVDV* and *CXCR4* mRNA data were produced from the "wash" experiment, and viral mRNA concentration increased in culture over time. Additionally, *CXCR4* mRNA concentration increased in infected PBMCs beginning at 32 hpi. This peak in CXCR4 *mRNA* concentration was interesting, and it warranted further investigation in subsequent experiments. However, the TCR pathway gene targets did not show any significant differences in this experiment. This might be the result of CXCR4 signaling through a different cascade pathway than what is presently recognized. Other targets were investigated in subsequent experiments.

*4.5 Pilot blocking experiments*

AMD3100 did not appear to interfere with BVDV RNA expression. However, the time course of the experiment was problematic in that viral growth did not occur until 72 hpi in previous experiments. Thus, we could not conclude that AMD3100 did not interfere with *BVDV* mRNA expression without extending the experiment through 72 hpi.

*IFN β* mRNA expression was also altered in the presence of AMD3100 concentrations. The highest increase in *IFN-β* mRNA expression was seen with 1.25 µg/mL AMD3100. Since an increase in IFN-I molecules in infected PBMCs was imperative for the *in vitro* model, this result helped us to select this concentration of AMD3100 for the expanded experiment. Also, the lower AMD3100 concentration (1.25 g/mL) was used in future experiments since it showed the most potential for having a negative modulatory effect on the virus *in vitro*. The control-treatment cohorts were removed from future experiments because the focus was whether AMD3100 1.25µg/mL concentration would interfere with viral mRNA expression. AMD3100 antagonization of CXCR4 prompted analysis of the modulatory effects of CXCR4's natural ligand, CXLC12, on BVDV infection. A single concentration of AMD3100 and CXCL12 was used in addition to a combination of the reagents. This experimental design was performed to identify a relationship between antagonizing/agonizing the receptor and viral mRNA expression.

## CHAPTER III: IFN-I AND CXCR4 EXPRESSION IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS INFECTED WITH NCP-2 96b2222 BVDV

### 1. Introduction

We hypothesized that infection of PBMCs with ncp-2 96b2222 BVDV *in vitro* will mount an IFN-I response and be mediated through and increase CXCR4 and CXCL12. Bovine viral diarrhea virus causes morbidity in cattle herds in the United States and worldwide (Ridpath et al., 1994). This may be attributed to the difficulty in eradicating the virus from herds due to challenges in identifying animals persistently infected (PI) with the virus.

Fetuses PI with ncp-2 96b2222 BVDV strain have a subtle but chronic IFN-I response (Shoemaker et al., 2009). Data from our laboratory have demonstrated a pronounced IFN-I, interferon-stimulated gene 15 (ISG15), and antiviral molecule (RIG-I) induction *in vivo* (Shoemaker et al., 2009; Smirnova et al., 2008). Additionally, a molecule novel to the study of BVDV, chemokine receptor four (CXCR4) was identified using microarray analysis. In this study, heifers carrying PI fetuses had diminished peripheral blood cell CXCR4 mRNA at 160 days gestation when compared to controls (Smirnova et al., 2009).

Chemokines are 8-10 kD molecules that are highly chemotactic for leukocytes. Chemokines are classified into four different groups based on the location of the first one

or two cysteine residues (Busillo and Benovic, 2007; Di Salvo et al., 2000; Nagasawa et al., 1998; Volin et al., 1998). Chemokine receptors are G-protein-coupled receptors and couple to pertussis-sensitive Gi proteins (Busillo and Benovic, 2007). CXCR4, a 352 amino-acid rhodopsin-like G-protein-coupled receptor (GPCR), is highly expressed in the thymus, particularly in immature CD4+ and CD8+ cells (Zou et al., 1998). In general, the chemokine-chemokine receptor relationships are promiscuous (Busillo and Benovic, 2007). However, CXCR4 and its ligand, chemokine ligand 12 (CXCL12), are considered quite monogamous (Amara et al., 1997; Busillo and Benovic, 2007; Jones and Nelson, 2007; Kumar et al., 2006; Maekawa and Ishii, 2000; Sodhi et al., 2004; Zou et al., 1998), although CXCL12 has recently been shown to bind receptors other than CXCR4 (Hatse et al., 2006). CXCL12 is constitutively secreted by marrow stromal cells (Burger and Burkle, 2007; Burger and Peled, 2009).

Further, chemokines can act during inflammation to attract inflammatory cells or during homeostasis to coordinate cell trafficking (Burger and Peled, 2009). Notably, CXCR4 has been implicated in a number of developmental processes, including hematopoiesis, angiogenesis, and organogenesis (Busillo and Benovic, 2007; Maekawa and Ishii, 2000; Zou et al., 1998). Also, CXCR4 has been implicated in a wide range of virally-induced diseases, and it has been studied extensively in HIV infection (Sodhi et al., 2004; Volin et al., 1998); for example, it has been shown that CXCR4 acts as a co-receptor with CD4 and facilitates HIV-viral entry (Maekawa and Ishii, 2000; Sodhi et al., 2004), initiating interactions between the viral envelope glycoprotein gp120 and CD4 (Sodhi et al., 2004). GPCRs can transmit pro- or anti-apoptotic signals, and use of CXCR4 by HIV has been shown to trigger CD4 depletion, although the mechanism is

unknown (Sodhi et al., 2004). Blocking CXCR4 with small ligands, such as AMD3100, effectively interferes with HIV entry (Zou et al., 1998). The mechanism by which AMD3100 binds to CXCR4 is well-described; it has been shown to also initiate a rapid and transient leukocytosis due to mobilization of hematopoietic cells into the blood from the bone marrow *in vivo* (Burger and Peled, 2009). Unlike binding CXCR4 with AMD3100, the interaction with CXCL12 is functional; CXCL12 is targeted to the endoplasmic reticulum to inactivate CXCR4 transcription, which results in a down-regulation of CXCR4 expression (Zhang et al., 2009). Others have shown that recombinant manipulation of CXCL12 can lead to a decrease in viral entry via a down-regulation in CXCR4 (Zhang et al., 2009). Additionally, it is postulated that a down-regulation of CXCR4, mediated by CXCL12, may be an antiviral principle (Amara et al., 1997). However, the role of CXCR4 during pestivirus infection, its relationship to IFN-I and IFN-I-stimulated genes, and cellular entry has not been explored.

IFN-I responses were investigated through application of an *in vitro* model with the intent to better understand the *in vivo* innate immune response in BVDV infection. An association between CXCR4 expression and the innate immune response against BVDV has not been investigated. However, our laboratory has noted an increase in CXCR4 expression in PI fetal blood (unpublished data), while Smirnova et al. found down-regulation of CXCR4 expression in animals carrying PI fetuses (Smirnova et al., 2009). These maternal data are contrary to the PI fetal data, which might suggest that the maternal change is a response to the PI fetus during its development.

To examine the PI immune response and investigate CXCR4 expression, an *in vitro* model for BVDV infection was developed with bovine peripheral blood mononuclear cells (PBMCs). PBMCs sustained in cell culture and infected *in vitro* should have the capacity to produce IFN-I and express CXCR4, two findings that our laboratory has documented in PI animals *in vivo*. PBMCs were used instead of a bovine-derived cell line (ATCC CRL#6017) because PBMCs produced detectable levels of viral mRNA (see Chapter 2, Figure 2.1).

We hypothesized that infection of PBMC with ncp-2 96b2222 BVDV *in vitro* will mount an IFN-I response and be mediated through and increase CXCR4 and CXCL12. Experiments examined if infection of PBMCs infected with ncp-2 96b2222 BVDV 1) induced IFN-I response; 2) induced CXCR4 and CXCL12 mRNA; 3) was enhanced by CXCL12 and mediated through CXCR4; and 4) affected immune cell populations.

## 2. Materials and Methods

### *2.1 In vitro infection*

Blood was collected from a BVDV-naïve steer via the jugular vein into sodium-EDTA-coated collection tubes. PBMC were isolated using Histopaque 1077 (For details, see Chapter 1, 2.2). Cells were counted and 2 x $10^6$ cells/mL were suspended in RPMI-1640 (1%Penicillin/Streptomycin/Amphotericin B) and 10% certified BVDV-free FBS (for details, see Chapter 1, 2.2). Cells were plated in triplicate into 12-well plates with 1 mL cell suspension (2 x $10^6$ cells/well) or 24-well plates with 0.25 mL cell suspension (5 x $10^5$ cells/well) for incubation and blocking experiments. Cells were incubated overnight at 37°C and 5% $CO_2$. Twenty-four hours later, cells were infected with ncp-2 96b2222

BVDV at a multiplicity of infection (moi) of 1 and sampled at either six, eight, or 12-hour intervals post-infection. An moi of 1 was used after optimization experiments identified it as the most appropriate for this viral strain during *in vitro* experiments (see Chapter II, Figure 2.4). Cell viability was assessed at each collection timepoint.

*2.2 RT-PCR*

Cell samples were collected into TRI-reagent (Sigma, USA). RNA was isolated per manufacturer's instructions, cleaned up by using the RNeasy MinElute cleanup kit (Qiagen, USA) and quantified on the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) constructed cDNA. *BVDV, IFN-B, ISG15, RIG-I, CXCR4, CXCL12, CD4, CD8, CD14, MHC-I,* and *MHC-II* were target genes for RT-PCR. Amplification was conducted on the LightCycler480 (Roche, Basal, Switzerland) with iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). Each reaction was carried out in duplicate and contained 2$\mu$L of cDNA template, 1.5 $\mu$L of a primer master mix containing 10$\mu$L each of 100uM forward and reverse primers diluted 1:13 in RNAse free water, 5$\mu$L of Supermix, and 1.5 $\mu$L RNAse-free water. The PCR reaction was carried out using the following protocol: 30s denaturation at 95°C, 30s annealing at 65°C, and 15s elongation at 70°C for 40 cycles. Fluorescence data were collected during all 40 cycles. PCR products were evaluated by gradual decreasing of temperature from 95°C to 55°C and collecting melt curve data for 8 minutes. Ct values were obtained from the Lightcycler software and analyzed in reference to *GAPDH* Ct values for each sample to determine $\Delta$Ct and relative expression of targets. Data were quantified as $2^{-\Delta Ct}$, and infected cell

values were expressed as a fold change compared to control values, where applicable. (See Table 1).

*2.3 Flow cytometry*

Cells were incubated in 24-well plates as described above. Cells were harvested and re-suspended in flow buffer, containing phosphate buffered saline (PBS) and 2% FBS. Cells were stained with antibodies for surface antigens in the described dilutions (Table 3) and incubated in the dark at room temperature for 30 min: CD4-FITC (AbD Serotec, CA; MCA1653F); CD8-FITC (AbD Serotec, CA; MCA837F); CD14-FITC (AbD Serotec, CA; MCA2678F); CXCR4-PE (BD Pharmingen; Cat # 557145). Cells were processed for BVDV intracellular staining, however the process did not yield reliable results (data not shown). Cells were washed in PBS two times, fixed in FACS fixative (PBS with 1% paraformaldehyde), and analyzed on the CyanADP flow cytometer (DAKO, CO) using Summit v4.3 software. Optimization dilutions were determined for compensation and surface antigen staining (Table 2 A-B).

*2.4 Blocking Experiments: AMD3100, CXCL12, AMD3100 and CXCL12*

Cells were incubated overnight as described above. Prior to virus infection, 1.25 μg/mL AMD3100, 0.25 μg/mL CXCL12 (Peprotech, NJ), or 1.25 μg/mL AMD3100 and 0.25 μg/mL CXCL12 was added to each well and incubated with cells for 15 minutes (Smirnova et al., 2005). Virus was added to the wells at moi 1 for one hour and cultured as described previously for 60 minutes at 37°C and 5% $CO_2$. Cells were washed and media containing a final concentration of 1.25 μg/mL AMD3100, 0.25 μg/mL CXCL12

(Peprotech, NJ), or 1.25 μg/mL AMD3100 and 0.25 μg/mL CXCL12 was added to each well for the time course of the experiment.

**Table 2. Optimization Dilutions for Bovine Antibodies: A) Compensation**

| A) | Cells | FITC Compensation | PE Compensation | APC Compensation |
|---|---|---|---|---|
| FITC | X | CD4: 10 μL | | |
| PE | X | | CXCR4: 25 μL | |
| APC | X | | | CD45: 10 μL |

**Table 2. Optimization Dilutions for Bovine Antibodies: B) Surface Antigen Staining**

| B) | CD45 CXCR4 | BVDV CXCR4 | T cytotoxic | T helper | Monocytes |
|---|---|---|---|---|---|
| FITC | | | CD8 10 μL | CD4 10 μL | CD14 10 μL |
| PE | CXCR4 25 μL | CXCR4 25 μL | CXCR4 25 μL | CXCR4 25 μL | CXCR4 25 μL |
| APC | CD45 10 μL 1:100 | BVDV i/cell 1μL 1:50 | BVDV i/cell 1μL 1:50 | BVDV i/cell 1μL 1:50 | BVDV i/cell 1μL 1:50 |

*2.5 Statistical Analysis*

The RT-PCR data (ΔCt values) were analyzed using the Student's t-test. Differences were described as $P < 0.05$. RT-PCR data were presented as $2^{-\Delta Ct}$. Flow cytometry was analyzed with a Dunnett's t-test, and homogeneity was assessed with the Levene's test for homogeneity. On occasions when the Dunnett's t-test was not appropriate, the Kruskal-Wallis test was used. For the Dunnett's t-test, α was set at $P=0.10$ to describe tendencies.

**3. Results**

*3.1 BVDV, IFN-β, ISG15, RIG-I, CXCR4 and CXCL12 mRNA Expression*

*BVDV* mRNA concentration increased ($P < 0.05$) in culture over time (Figure 3.1a). By 72 hpi, cell viability was >85%. *IFN-β* mRNA concentration increased in virus-treated PBMC when compared to controls at 32, 36, and 72 hpi ($P < 0.05$) and 48 hpi ($P < 0.01$) (Figure 3.1 b). Similarly, *ISG15* mRNA expression increased in virus-treated PBMC at 32, 48 and 72 hpi ($P < 0.05$) compared to controls (Figure 3.1 c). *RIG-I* mRNA expression increased 32 and 60 hpi ($P < 0.05$) and 48 hpi ($P < 0.01$) (Figure 3.1 d).

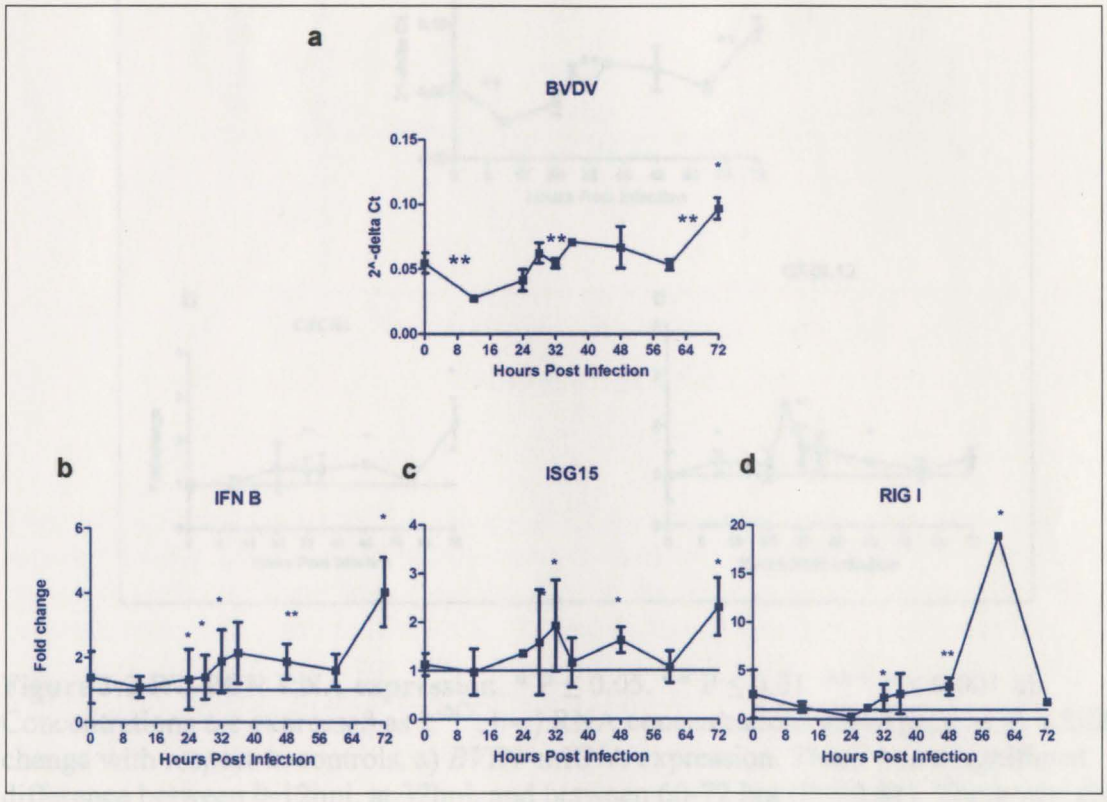


**Figure 3.1. RT-PCR RNA expression.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ a) Concentrations are expressed as $2^{-\Delta Ct}$. b-d) Concentrations are expressed as a fold change with respect to controls. a) *BVDV* mRNA expression. There was a significant difference between 0-12hpi, at 32hpi, and between 60-72 hpi ($P < 0.01$). There was also a significant difference between 0-72 hpi ($P < 0.05$) b) *IFN β* mRNA expression. There was a significant difference between control and virus-treated PBMCs at 24, 28, 32, and 72 hpi ($P < 0.05$) and 48 hpi ($P < 0.01$). c) *ISG15* mRNA expression. There was a significant difference between control and virus-treated PBMCs at 32, 48 and 72 hpi ($P < 0.05$). d) *RIG-I* mRNA expression. There was a significant difference between control and virus-treated PBMCs at 32 and 60 hpi ($P < 0.05$) and 48 hpi ($P < 0.01$).

*CXCR4* mRNA expression increased in virus-treated PBMCs at 32, 48 ($P<0.01$) and 72 ($P < 0.05$) hpi compared to controls (Figure 3.2b). *CXCL12* mRNA expression increased in virus-treated PBMCs at 12, 36 48 hpi ($P < 0.05$) and 32 hpi ($P < 0.01$) compared to controls (Figure 3.2 c).

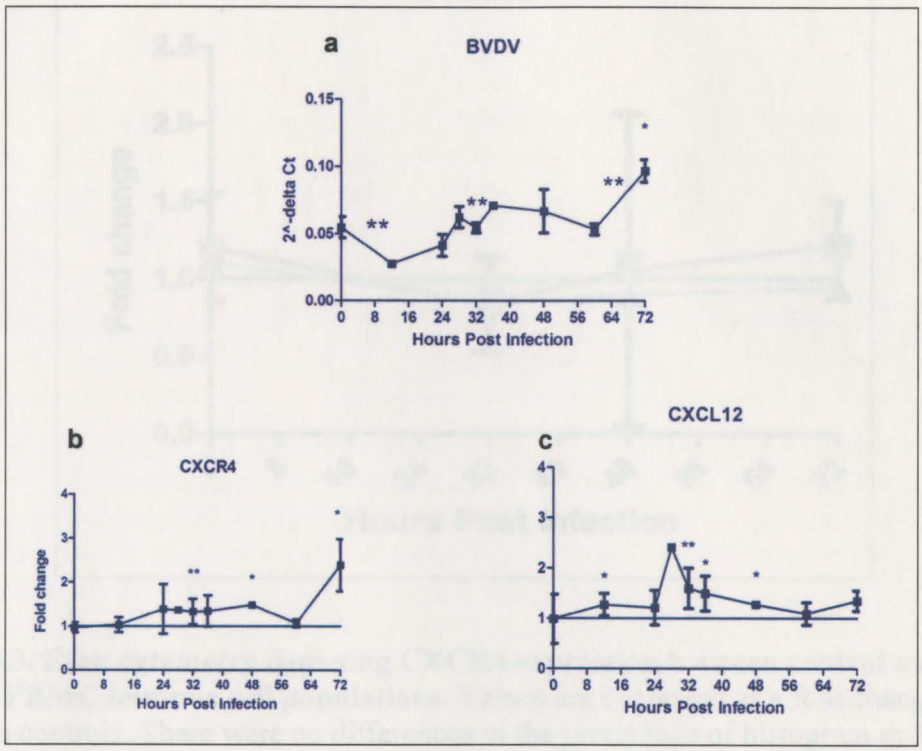


**Figure 3.2 RT-PCR RNA expression.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$ a) Concentrations are expressed as $2^{-\Delta Ct}$. b-c) RNA concentrations are expressed as a fold change with respect to controls. a) *BVDV* mRNA expression. There was a significant difference between 0-12hpi, at 32hpi, and between 60-72 hpi ($P < 0.01$). There was also a significant difference between 0-72 hpi ($P < 0.05$) b) *CXCR4* mRNA expression. There was a significant difference between control and virus-treated PBMCs at 32 ($P < 0.01$) and 48 and 72 hpi ($P < 0.05$). C) *CXCL12* mRNA expression. There was a significant difference between control and virus-treated PBMCs at 12, 36 and 48 hpi ($P < 0.05$) and 32 hpi ($P < 0.01$).

*3.2 Flow cytometry: CXCR4 expression*

CXCR4 expression increased ($P < 0.10$) in infected PBMCs compared to controls at 72 hpi (Figure 3.3). The MFI data appeared to be down-regulated in infected cells compared to control, however the data were not statistically different.

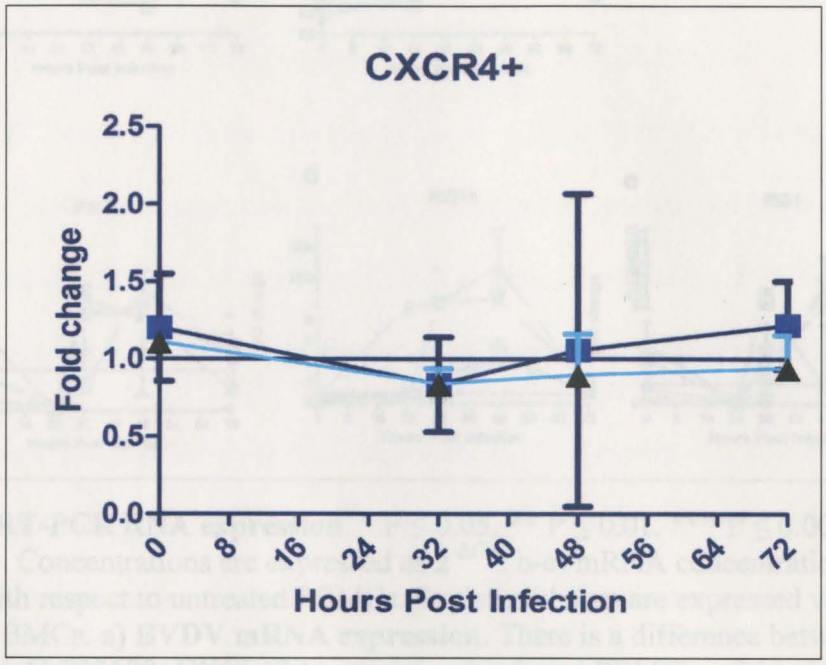


**Figure 3.3. Flow cytometry depicting CXCR4 expression between control and infected PBMC immune cell populations.** Values are expressed as a fold change with respect to controls. There were no differences in the percentage of histogram or MFI between control and infected PBMCs. The percentage of histogram appeared to be increased infected PBMCs compared to controls at 72 hpi. The MFI appeared to be decreased in infected PBMCs at 32, 48 and 72 hpi.

*3.3 Blocking experiments: mRNA expression and flow cytometry*

In the presence of AMD3100, *BVDV* mRNA expression was not significantly different compared to untreated infected PBMCs (Figure 3.4a). However, *BVDV* mRNA concentration was increased ($P < 0.05$) compared to controls at 24 hpi when treated. Further, AMD3100 abrogated the effects of CXCL12 on viral mRNA expression.

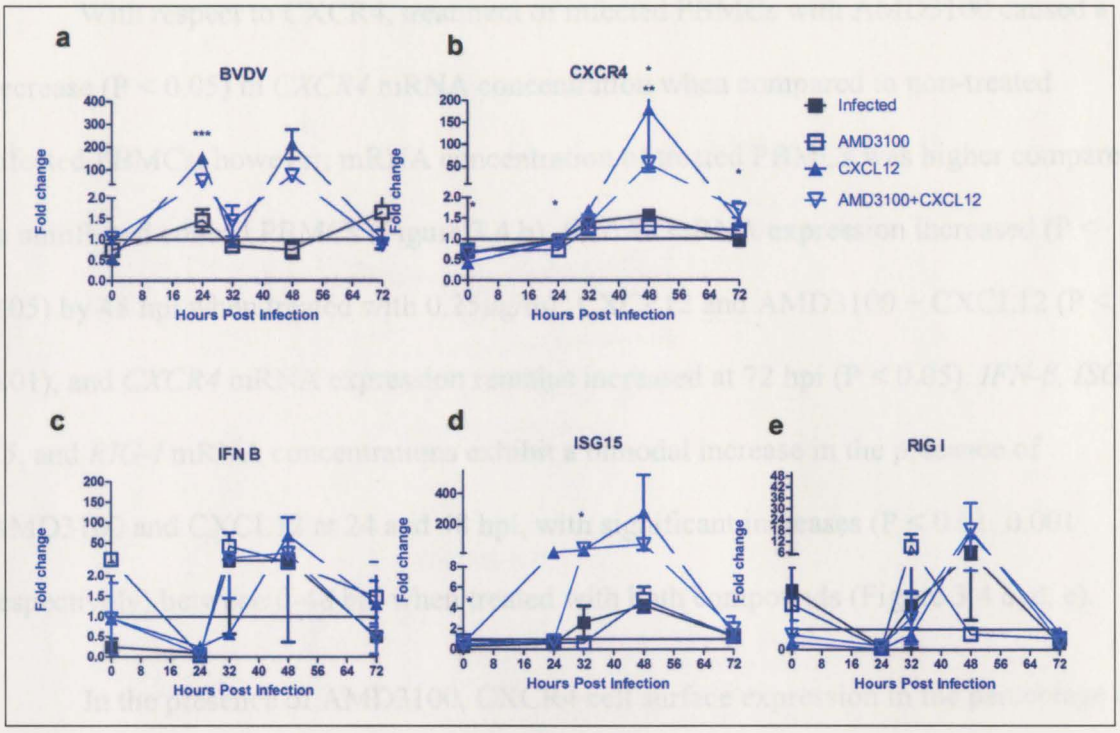


**Figure 3.4. RT-PCR RNA expression.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$. x=*. y=**. z=***. Concentrations are expressed as $2^{-\Delta Ct}$ . b-e) mRNA concentrations are expressed with respect to untreated PBMCs. Statistics shown are expressed with respect to infected PBMCs. **a) BVDV mRNA expression**. There is a difference between CXCL12 and AMD3100+CXCL12 treatment and infected PBMCs at 24 hpi ($P < 0.001$). Differences are calculated, but asterisks not shown: Infected cells: (x) between 0-32, 0-72 hpi. AMD3100 treatment: (x) between 0-24, 0-32, 0-48, (z) between 0-72. CXCL12 treatment: (z) between 0 hpi and all timepoints. AMD3100+CXCL12 treatment: (x) between 0-32 hpi, and (z) between 0-24, 48 and 72 hpi. **b) CXCR4 mRNA expression**. There is a decrease in *CXCR4* mRNA in PBMCs treated with CXCL12 at 0hpi ($P < 0.05$) and AMD3100 at 24 hpi ($P < 0.05$) compared to untreated infected PBMCs. There is an increase in *CXCR4* mRNA concentration in cells treated with CXCL12 ($P < 0.05$) and AMD3100 + CXCL12 ($P < 0.01$) at 48 hpi and PBMCs treated with AMD3100 and AMD3100 + CXCL 12 ($P < 0.05$) at 72 hpi. Differences are calculated, but asterisks not shown: Infected cells: (y) 0-48; AMD3100 treatment: (x) significance at 32 hpi, (y) 0-24, 32, 72 hpi; CXCL12 treatment: (z) 0-48 hpi; AMD3100 + CXCL12 treatment: (x) between 0-24, 32 hpi (y) 0-72, 72 hpi, (z) 0-72 hpi. **c) IFN B mRNA expression**. There were no differences between untreated infected PBMCs and treated PBMCs. Differences are calculated, but asterisks not shown: AMD3100, AMD3100+ CXCL12 treatment: (y) 0-48 hpi. **d) ISG 15 mRNA expression**. There is an increase in ISG15 RNA concentration in PBMCs treated with AMD3100 + CXCL12 ($P < 0.05$) at 0 and 32 hpi. Differences are calculated, but asterisks not shown: CXCL12 treatment: (y) 0-48. AMD3100 + CXCL12: (z) 32, 0-48 hpi. **e) RIG-I mRNA expression**. There were no differences between untreated infected PBMCs and treated PBMCs. Differences are calculated, but asterisks not shown: AMD3100 + CXCL12 treatment: (y) 0-48 hpi.

With respect to CXCR4, treatment of infected PBMCs with AMD3100 caused a decrease ($P < 0.05$) in *CXCR4* mRNA concentration when compared to non-treated infected PBMCs; however, mRNA concentration of treated PBMCs was higher compared to uninfected control PBMCs (Figure 3.4 b). *CXCR4* mRNA expression increased ($P < 0.05$) by 48 hpi when treated with 0.25$\mu$g/mL CXCL12 and AMD3100 + CXCL12 ($P < 0.01$), and *CXCR4* mRNA expression remains increased at 72 hpi ($P < 0.05$). *IFN-β, ISG 15*, and *RIG-I* mRNA concentrations exhibit a bimodal increase in the presence of AMD3100 and CXCL12 at 24 and 48 hpi, with significant increases ($P < 0.01$, 0.001 respectively) between 0-48 hpi when treated with both compounds (Figure 3.4 c, d, e).

In the presence of AMD3100, CXCR4 cell surface expression in the percentage of histogram and MFI had the tendency to be decreased ($P < 0.10$) (Figure 3.5 a, b). The MFI of CXCR4 had the tendency to be decreased in the presence of CXCL12 at 48 hpi as well as for AMD3100 and AMD3100 + CXCL12 treatments at 72 hpi ($P < 0.10$). CXCL12 treatment appeared to increase CXCR4 cell surface expression in the percentage of histogram, but data are not significantly different.

*3.4. Immune cell markers: mRNA concentration and flow cytometry*

*CD4* mRNA expression was significantly increased in infected cells compared to controls at 0, 12, 24, 48 and 60 hpi ($P < 0.05$) (Figure 3.6 a). *CD8* mRNA expression was significantly increased in infected cells compared to controls at 12, 36, 48, 60, 72 hpi ($P < 0.05$) and at 32 ($P < 0.01$) (Figure 3.6 b). *CD14* mRNA concentration was decreased in infected cells compared to controls at 24, 48 hpi ($P < 0.05$) and at 72 hpi

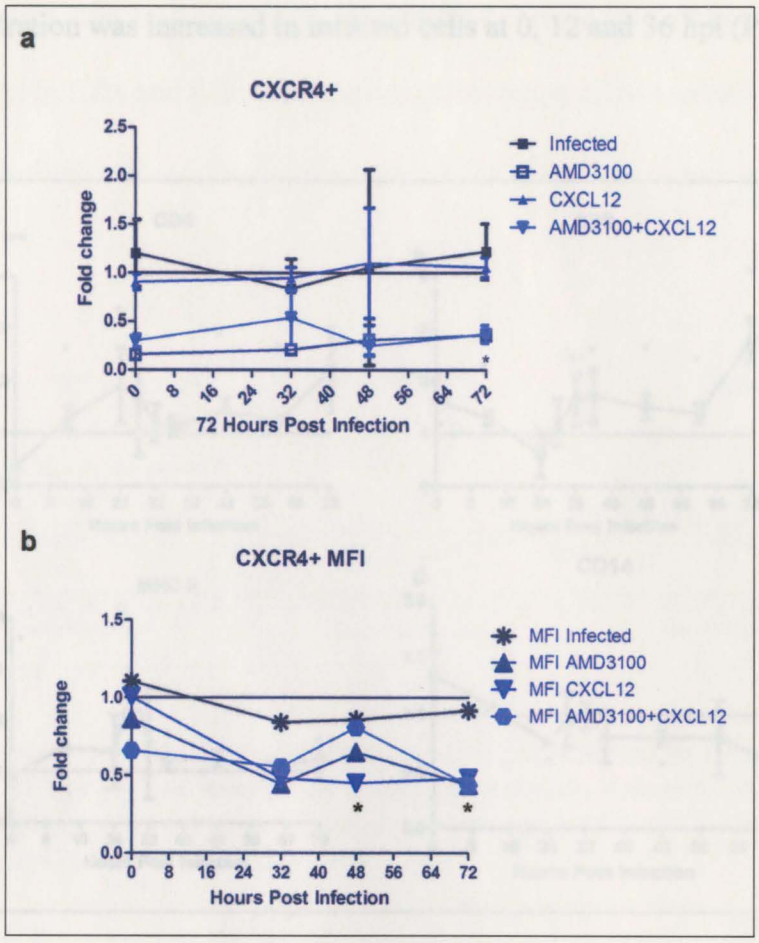


**Figure 3.5. Flow cytometry depicting CXCR4 expression in PBMCs treated with AMD3100, CXCL12, or both.** Values are expressed as a fold change with respect to controls. Statistical analysis reflects comparisons made against infected PBMC values. a) Percentage of histogram. There was a tendency for the number of cells that expressed CXCR4 to decrease in the presence of AMD3100 and AMD3100 + CXCL12: $P < 0.10$ at 72 hpi. B) Mean Fluorescence Intensity. Statistical analysis reflects comparisons against infected PBMCs. There was a tendency for the number of CXCR4 molecules per cell to decrease when treated with CXCL12 at 48 hpi ($P < 0.10$) and when treated with AMD 3100, CXCL12, AMD3100 + CXCL12 at 72 hpi ($P < 0.10$).

($P < 0.01$) (Figure 3.6 d). *MHC-I* mRNA expression was not significantly increased in infected cells compared to controls at any timepoints (data not shown), while *MHC-II*

mRNA concentration was increased in infected cells at 0, 12 and 36 hpi ($P < 0.05$) (Figure 3.6 c).

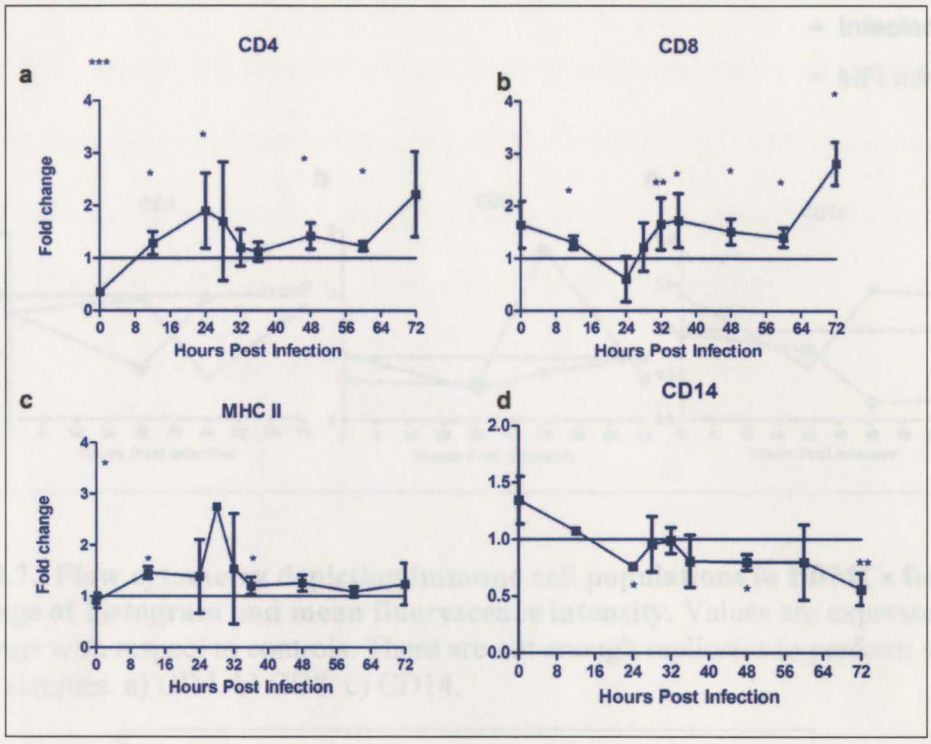


**Figure 3.6. RT-PCR RNA expression.** * $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$. mRNA concentrations are expressed as a fold change with respect to controls. a) Infected cells had higher mRNA concentrations at 0 ($P < 0.001$), 12, 24, 48 and 60 ($P < 0.05$) hpi. b) Infected cells had higher mRNA concentrations at 0, 36, 48, 60, 72 ($P<0.05$) and 32 ($P < 0.01$) hpi. c) Infected cells had lower RNA concentrations at 24, 48 ($P < 0.05$) and 72 ($P < 0.01$) hpi. d) Infected cells had higher mRNA concentrations at 0 ($P < 0.001$), 12, and 36 ($P < 0.05$) hpi.

There appeared to be higher numbers of cells in CD4 PBMC populations compared to CD8 and CD14 cells across all timepoints, with the highest numbers of cells at 32 and 72 hpi (Figure 3.7 a-c). There appeared to be an increase in the number of CD14+ cells in infected PBMCs at 72 hpi; CD4, CD8, and CD14 populations appeared to demonstrate higher cell counts in the control compared to infected PBMC populations at

all other timepoints. The MFI appeared to be the most increased at 48 hpi for CD8 and CD14 and 72 hpi in CD4 and CD14 in infected cells compared to controls.

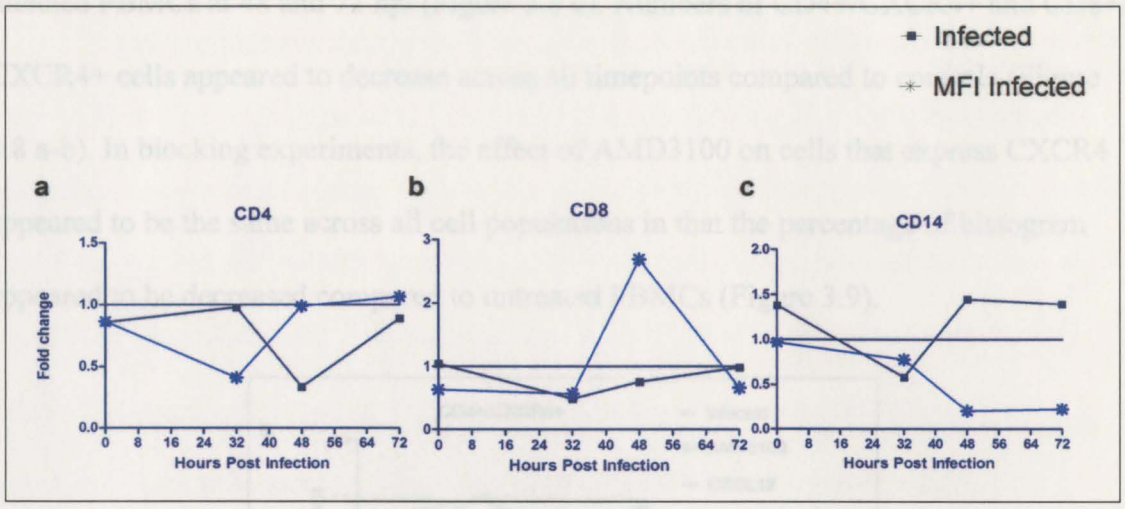


**Figure 3.7. Flow cytometry depicting immune cell populations in PBMCs for percentage of histogram and mean fluorescence intensity.** Values are expressed as a fold change with respect to controls. There are not enough replicates to perform statistics on these samples. a) CD4. b) CD8. c) CD14.

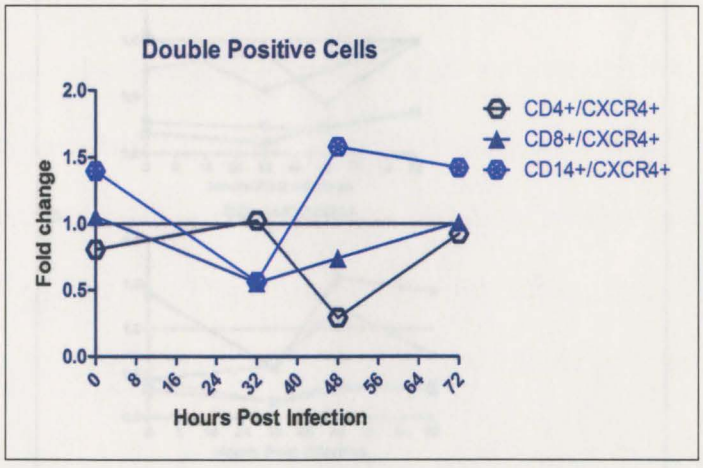


**Figure 3.8. Flow cytometry depicting CXCR4 expression in immune cell populations of infected PBMCs.** Values are expressed as a fold change with respect to controls. There are not enough replicates to perform statistics on these samples. a)CD4+/CXCR4+. b) CD8+/CXCR4+. c) CD14/CXCR4+. This sample is the only one in which CXCR4 expression is higher in infected PBMCs compared to controls. This occurs at 48 and 72 hpi.

Although there was a sample size of one for CD4, CD8, and CD14 surface antigen staining, there appeared to be more double positive CD14+/CXCR4+ cells in infected PBMCs at 48 and 72 hpi (Figure 3.8 c). Numbers of CD4+/CXCR4+ and CD8+/CXCR4+ cells appeared to decrease across all timepoints compared to controls (Figure 3.8 a-b). In blocking experiments, the effect of AMD3100 on cells that express CXCR4 appeared to be the same across all cell populations in that the percentage of histogram appeared to be decreased compared to untreated PBMCs (Figure 3.9).

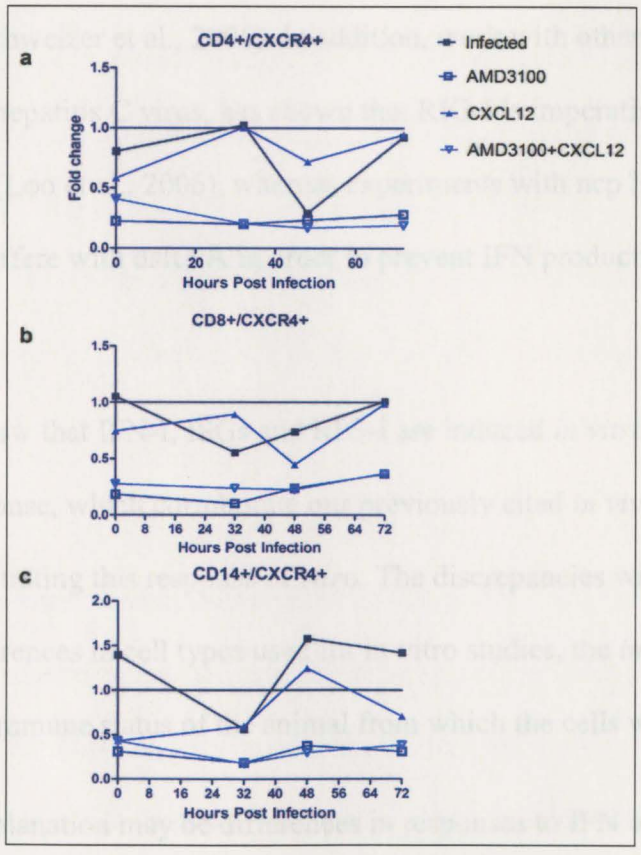


**Figure 3.9 Flow cytometry depicting CXCR4 expression in immune cell populations of PBMCs during blocking experiments**. Values are expressed as a fold change with respect to controls. Across all cell populations, AMD3100 appeared to decrease CXCR4 expression. a) CD4+/CXCR4+. b) CD8+/CXCR4+. c) CD14+/CXCR4+. CXCR4 expression appeared to be higher in infected PBMCs compared to controls at 48 and 72 hpi.

## 4. Discussion

### *4.1 IFN-I, ISGs, and RIG-I*

Other experiments have shown that persistent infection with ncp BVDV does not elicit an IFN-I response against the virus, but it does allow for IFN-I production and protection against other viruses (Schweizer et al., 2006). Further, pre-treatment of Madin-Darby bovine kidney (MDBK) cells with IFN-I allowed for a significant reduction in the amount of virus produced, whereas treatment with IFN-I after infection led to a much less drastic decrease (Schweizer et al., 2006). In addition, work with other pestivirus infections, such as hepatitis C virus, has shown that RIG-I is imperative during the induction of IFN-I (Loo et al., 2006), whereas experiments with ncp BVDV have shown that ncp strains interfere with dsRNA in order to prevent IFN production (Schweizer and Peterhans, 2001).

Our data show that IFN-I, ISGs and RIG-I are induced *in vitro* in bovine PBMC as an antiviral response, which corroborate our previously cited *in vivo* data. This is the only report demonstrating this response *in vitro*. The discrepancies with other literature may be due to differences in cell types used for in vitro studies, the *in vitro* activity of the viral strain, or the immune status of the animal from which the cells were harvested.

Another explanation may be differences in responses to IFN or cytokines within the infected cell, which also could depend on the viral strain. Different strains of BVDV may have different cellular tropisms, which may lead to the induction of different signal transduction pathways during infection, which is common in viral infections. For instance, viruses respond differently to different interferon molecules. Pretreatment of

PBMCs with IFN-I increases HIV binding and replication likely due to HIV-induced increase in expression of HIV receptors and co-receptors on the plasma membrane. In HIV infection, IFN-I leads to an increase in expression of CXCR4 and CD4 in PBMC (Serra et al., 2008). However, HeLa-T4, HT-29, and T-24 epithelial cells treated with type-2 interferon (IFN-II), IFN-gamma, have a decrease in CXCR4 expression and an increase in CCR5 in the face of M-tropic HIV (Biolchini et al., 2000). This demonstrates that epithelial co-receptors respond differently to IFN-gamma, suggesting differences in IFN effects depending on the viral strain. Beta chemokines such as MIP-1 alpha, MIP-1 $\beta$, and RANTES have been shown to allow for increased replication of T-cell tropic HIV infection in peripheral blood leukocytes via an increased expression of CXCR4, but not for M-tropic strains(Dolei et al., 1998) and that HIV adsorption and spread is mediated by HIV-induced cytokines (Dolei et al., 1994). Thus, it is plausible that a derangement or change in the IFN- or BVDV-induced cytokines may affect the induction of the antiviral pathway or the effectiveness and efficiency of the virus, dependent on strain and activity *in vitro*.

The specific immune response in PBMCs infected with ncp-2 BVDV may be originating from the fact that the virus was adsorbed for 60 minutes to allow enough time for viral entry, thereby allowing for stimulation of an immune response in these cells. It has been shown that BVDV adsorption and spread is mediated by BVDV-induced cytokines, which is an important factor in the pathogenesis of the virus (Dolei et al., 1994). Further, the expression peak at 32 hpi is interesting. This may be due to the time needed for viral entry into the nucleus, and/or differences to accrue in molecular levels of

these molecules via the TCR and JAK/STAT pathway (Shoemaker et al., 2009; Smirnova et al., 2009), leading to an induction of the antiviral state.

*4.2 CXCR4-CXCL12*

CXCR4 and CXCL12 have been extensively investigated in conditions related to embryogenesis and development, oncology, and viral infections. For instance, investigations into manipulation of the CXCL12-CXCR4 axis in tumor growth and leukemia has been done in efforts to interrupt the migration and dissemination of tumor cells during metastasis and migration for sites such as the bone marrow (Burger and Peled, 2009). For instance, T and B lymphocytes use CXCL12-CXCR4 to home to specific lymphoid microenvironments during development and immune surveillance (Burger and Peled, 2009). The CXCL12-CXCR4 axis is imperative as a migration mechanism, conserved across species, and fundamental for stem cell migration in multiple tissues for the embryo and the adult (Burger and Peled, 2009; Zhang et al., 2009). Manipulation of this axis *in vivo* may explain how differential migration of cells in the embryo is dependent on the cytokine milieu to regulate cell proliferation and retention; aberrations in this axis in the presence of virus may contribute to the developmental abnormalities typical of the PI phenotype, such as leukopenia and immune suppression.

From a functional standpoint, CXCR4- and CXCL12-deficient animals have been developed, although viable offspring tend not survive to parturition and these animals demonstrate lesions in hematopoietic cell trafficking that suggest a role in these processes for these molecules (Maekawa and Ishii, 2000; Zou et al., 1998). Relevant lesions have

been described in the mouse, although explicit knowledge of the homing process is lacking (Maekawa and Ishii, 2000).

To date, targeting the CXCR4-CXCL12 axis has not been investigated during bovine BVDV infection. We showed that there was a significant upregulation in *CXCR4* and *CXCL12* mRNA expression 32 hpi similar to the increases in *IFN-β, ISG15* and *RIG-I* mRNA concentrations. An association between IFN-I and CXCR4 has been documented in other viral infections (Serra et al., 2008). Given our laboratory's previous knowledge of IFN-I and CXCR4 expression aberrations during BVDV infection *in vivo*, examination of IFN-I and CXCR4 RNA concentrations *in vitro* was important. To the author's knowledge, this report is the first to show *in vitro* activity of CXCR4 with respect to IFN-I in bovine PBMCs infected with ncp-2 96b2222 BVDV. The specific mechanism for this induction needs to be further elucidated. However, the similarities in the expression of *IFN-I, ISGs*, and *RIG-I* over time in culture suggest a possible inflammatory relationship for CXCL12 and CXCR4 during bovine BVDV infection. As in HIV infection (Biolchini et al., 2000; Dolei et al., 1998; Dolei et al., 1994), chemokines may increase the production of BVDV transcripts, which may stimulate the CXCR4 receptor via an IFN-I signaling pathway, as is reported for other viruses (Audige et al., 2006).

The consequences of CXCR4 activity during BVDV infection, as well as the mechanism for IFN-induced CXCR4 expression, are unknown. As stated previously, others have shown that CXCR4 acts as a co-receptor for viral entry (Nagasawa et al., 1998; Zou et al., 1998), but results from our experiments with antagonistic and agonistic

molecules indicate that CXCR4 does not affect BVDV entry. This is the first report to state that CXCR4 does not appear to have a role in BVDV cellular entry.

Flow cytometry identified the expression of CXCR4 at the cell surface of three different immune cell populations. Interestingly, in other species, CXCR4 is shown to be highly constitutively expressed on CD4 and CD8 T cells (Liu and Dorovini-Zis, 2009; Payne et al., 2009). In this report, the percentages of cell surface expression are lower than what has been reported for humans. This may be attributable to a species difference in CXCR4 expression or less specific antibody binding in bovine animals. Additionally, CXCR4 cell surface expression was not different statistically in infected PBMCs despite the differences noted at the mRNA level by 72 hpi. The lack of difference in CXCR4 cell surface expression might be due to the time course of the experiment in that it was not long enough for changes in cell surface expression to develop, or it might be rooted in reactions to the intracellular virus such that differences in transcription of CXCR4 are prevented from occurring at the protein level.

*4.3 Blocking experiments*

The mechanism for pretreatment with small molecule inhibitors of CXCR4 entails binding of AMD3100 to CXCR4, which induces receptor internalization. CXCR4 binding to CXCL12 stimulates downstream pathways of the receptor, followed by internalization (Amara et al., 1997; Vila-Coro et al., 1999; Virelizier, 1999). CXCL12 induces a down-regulation of CXCR4 in a dose-dependent manner (Amara et al., 1997; Liu and Dorovini-Zis, 2009). Blocking CXCR4 with AMD3100 led to a down-regulation of *CXCR4* mRNA and cell surface concentrations, and it ultimately abrogated a BVDV-

induced *CXCR4* upregulation. In the present studies, AMD3100 did not interfere with BVDV entry despite its ability to do so in other viral infections. Recent work has shown that chemokine receptor internalization may occur via clathrin-mediated endocytosis (Neel et al., 2005). Similarly, BVDV viral entry has been postulated to occur via a similar mechanism (Lecot et al., 2005) or by dependence on heterodimer formation of the viral envelope proteins E1/E2 (Ronecker et al., 2008). It is plausible that viral entry and chemokine receptor entry occur in a similar manner, and it may be another mechanism by which the virus subverts the host immune response. However, we were unable to demonstrate that AMD3100 interfered with BVDV entry into PBMCs.

Binding CXCR4 with CXCL12 led to an up-regulation at the mRNA level. In other viral infections, CXCL12 has been shown to modulate infection, such as in FIV (Hosie et al 1997; Regetti 2008). In these experiments, CXCL12 treatment appeared to facilitate the expression of *BVDV* and *CXCR4* mRNA, which supports other reports that CXCL12 promotes viral infection. Again, this emphasizes the importance of the CXCL12-CXCR4 axis during *in vitro* BVDV infection. CXCL12 treatment also led to an up-regulation of *IFN-β, ISG 15* and *RIG-I* RNA concentrations. This may suggest that, if CXCR4 is induced by IFN-I, it might respond in a negative feedback loop. That is, a decrease in *CXCR4* expression via internalization by binding CXCL12, as seen at 24 hpi, might lead to an increase in *IFN-β, ISG15* and *RIG-I* expression to stimulate an increase in CXCR4 expression via recycling back to the cell surface. These data might further support a potential relationship between IFN-I and CXCR4. Alternatively, these might be responses to viral replication cycles and represent 2 cycles of viral replication within

the cell. Using different concentrations of AMD3100 or CXCL12, a different time course of infection, or a different viral strain may alter the interpretation of these results.

On the cell surface, there was a decrease in CXCR4 expression in PBMCs treated with AMD3100, which corroborates mRNA concentration results, and both AMD3100 and CXCL12, which is contradictory to the mRNA concentration results. The decrease in CXCR4 cell surface expression might be because the AMD3100 and CXCL12 interfered with antibody binding sites, leading to a falsely-reported decrease in CXCR4. This would explain why there is an increase in CXCR4 at the mRNA level and a decrease at the cell surface level in PBMCs treated with both AMD3100 and CXCL12. Thus, the decrease in CXCR4 cell surface expression may be a reflection of cell surface inhibition and not a direct reflection of the decreases seen in CXCR4 transcription.

*4.4 Immune cell populations*

One aim was to determine CD4, CD8 and CD14 cell population distributions in the presence or absence of BVDV and to determine which cells expressed CXCR4. Although *CD4, CD8*, *CD14*, and *MHC II* mRNA concentrations differed in infected cells compared to controls, it is difficult to draw more definitive conclusions from the data presented. In order to better identify cell populations that are expressing these molecules, sorting cells first via flow cytometry and then collecting samples for RNA might serve to better identify these populations. However, we showed that *CD4, CD8*, and *MHC II* mRNA expression are all increased while *CD14* is decreased in infected PBMCs compared to controls. One reason for this increase in transcription of the *CD4, CD8,* and *MHC II* immune cell markers might be that these molecules are responding to initial

infection with efforts to produce more protein on the cell surface. These molecules have a functional role in the cellular immune response and are important for cellular differentiation, and so changes in cell surface expression might take longer to occur. Extending the time course of the experiment and evaluating the expression of these molecules later during infection might identify differences. Another reason for increases in mRNA concentrations of *CD4* and *CD8* might be that there is an interference with the translation of mRNA into protein for these molecules due to the presence of intracellular virus. Although cell viability was assessed at all time points, CD14 cells appeared to be increased in infected PBMC samples over time which might be a reflection of more rapid die-off of CD4/8 populations compared to CD14 cells. Cell cultures were not supplemented with compounds such as IL-2, and so these conditions may not be amenable to long-term survival for certain immune cell populations.

On the cell surface, CD14 molecules appeared to be increased at 48 and 72 hpi in infected PBMCs compared to controls, whereas CD4 and CD8 molecules appeared to be decreased in infected PBMCs across all time points. This is contrary to the mRNA results which demonstrated a significant increase later in infection in *CD8* mRNA expression in infected PBMCs and a decrease in *CD14* expression. One reason for this might be that the virus interferes with the translation of the molecule, its transport to the cellular membrane, or the overall effectiveness of the CD8 molecule. Additionally, other reports document no change in flow staining in leukocyte subpopulations in PI or non-PI animals (Brewoo et al., 2007). Our contrary results may be attributable to the different viral strain or tropism or that our PBMCs are acutely infected with the virus. Further, our data showed that MFI appeared to be increased in infected PBMCs compared to controls at 48

hpi (CD8, CD14) and 72 hpi (CD4, CD14), which might indicate that there are more PBMCs expressing CD14 at multiple time points after infection. These results might also be explained by a compensatory response by the CD14 PBMCs to the changing levels of CD4 and CD8 within the *in vitro* conditions. The data collected from double positive cells appeared to demonstrate a similar increase; the number of CD14 cells that express CXCR4 appeared to be increased compared to the other populations. This might indicate that CD14 cells express more CXCR4 and therefore might be more important immune response cells during ncp-2 96b2222 BVDV infection *in vitro*. However, the number of replicates in the experiment was limited because the experimental design was directed by the number of cells that were initially isolated from a single blood collection. Therefore, the likelihood for substantial variability cannot be ruled out within these results. To address this, additional information is needed and this experiment should be repeated on a larger scale to ensure statistical differences.

Others have shown *in vitro* that BVDV induces a type-I CD8 T-cell response and a type-2 CD4 T-cell response in PBMCs (Rhodes et al., 1999); such Th2 responses may avoid aggressive cellular responses and placental damage, thereby reducing the risk of abortion *in vivo*. During acute infection, Collen et. al. showed a depletion of CD4 cells but not CD8 or $\gamma/\Delta$ T-cells, suggesting a role for CD4s as effector or helper cells during infection (Collen and Morrison, 2000). In this report CD4 and CD8 T-cells were also primed by BVDV infection and different cell types responded differently to ncp and cp strains, cross reacted between serologically different viruses, and were MHC-restricted (Collen and Morrison, 2000). However, others have shown that depletion of CD4 cells in gnotobiotic calves infected with ncp BVDV led to extension of the viremia and higher

titers compared to CD8 depletion, suggesting a more pivotal role for CD4 cells during acute infection (Howard et al., 1992). As in other viral infections, early or acute infection leads to the expression of mainly ISGs, a gene profile which is dependent on IFN-α production by specific cell types and allows CD4 T-cells to limit viral replication (Audige et al., 2006). Although our data do not definitively suggest this, they may result via similar mechanisms such that the host immune response is able to interact with BVDV. Additionally, this relationship might suggest a collaborative or inhibitory role for CXCR4 with different immune cell markers during BVDV-infection *in vitro*.

Although others have shown that CD8 does not seem to be a major player in acute BVDV infection *in vivo* (Howard et al., 1992), Beer et. al. demonstrated the effective cytotoxic effects of CD8 cells *in vitro*. MHC-restriction in cytotoxic T-lymphocytes has also been described (Beer et al., 1997). Although our RT-PCR data indicate a difference between control and infected cells for *CD8* on the mRNA level, the flow cytometry data demonstrate a decrease of the CD8 molecule on the cell surface of infected PBMCs compared to control. Although our data do not definitively suggest this, the virus might act to shift the host cellular immune response from favoring a CD8 /Th1 response to a CD4/ Th2 response, which leads to a down-regulation of MHC-II and a decrease in CXCR4 expression. Additionally, the virus may act to shunt the production of CD4 and CD8 molecules, whether at the mRNA or protein level, to favor CD14 cells. Whether this is beneficial for the virus or the host is unknown at this time, and additional experiments are needed in order to further distinguish the activity of immune cells during ncp-2 96b2222 BVDV infection *in vitro*.

## CHAPTER IV: CONCLUSIONS

These chapters describe development of an *in vitro* model for ncp-2 BVDV infection. Optimizing conditions for the specific viral strain prior to performing larger-scale experiments was essential in order to glean the most useful data from each experiment. We concluded that infection of bovine PBMCs infected at moi 1 ncp-2 96b2222 BVDV followed by 60 minutes adsorption with the virus and replacement with fresh culture media sans virus yielded the most relevant data.

A number of questions were examined in order to recapitulate *in vitro* results that were previously described during PI *in vivo*: 1) is there an IFN-I response in PBMCs infected with ncp-2 96b2222 BVDV? 2) does blocking CXCR4 with the synthetic antagonist AMD3100 or natural ligand CXCL12 prevent ncp-2 96b2222 BVDV entry into PBMCs? 3) is there a difference in CXCR4 expression levels between control and infected PBMC across immune cell populations?

Increases in *IFN-I, ISG15*, and *RIG-I* mRNA expression levels in bovine PBMCs occurred *in vitro* after infection with ncp-2 96b2222 BVDV, suggesting an IFN-I response. An association between IFN-I and CXCR4 has been documented in other viral infections. Increases in *CXCR4* and *CXCL12* mRNA expression occurred at similar times pi, suggesting that CXCR4 may mediate the antiviral state during acute infection with ncp-2 BVDV *in vitro*. To the authors' knowledge, this is the first demonstration of a

BVDV-induced up-regulation of *CXCR4* expression. CXCR4 cell surface expression appeared to increase when examined with flow cytometry methodology, however small sample sizes prevented differences between groups from being statistically different. AMD3100 blocked BVDV-induced upregulation of *CXCR4* mRNA, noted via RT-PCR, and the tendency for CXCR4 cell surface expression to decrease were also noted via flow cytometry though the presence AMD3100 and/or CXCL12 did not appear to prevent viral entry. Finally, an examination of cell population distributions with flow cytometry appeared to indicate that CD14+ cell population is increased in infected cells, and there might be a difference in expression of immune cell populations in infected PBMCs. The data also appeared to indicate that CXCR4 cell surface expression was increased in bovine CD14+ cells compared to CD4+ and CD8+ at 72 hpi.

This is the first report to evaluate the role of CXCR4 transcription during BVDV infection *in vitro*. The described increase in CXCR4 expression in infected PBMCs in response to CXCL12 treatment is a significant finding because CXCL12 has been shown to modulate infection for other viral agents. The roles of CXCR4 and CXCL12 during pestivirus infection should be investigated *in vitro* and *in vivo*. Additional experiments suggested by findings described here include: 1) evaluation of the effect of pretreatment of PBMCs with IFN-I prior to infection with ncp-2 96b2222 BVDV on CXCR4 expression, which might address any potential association between IFN-I and CXCR4 expression; 2) examination of a dose-range of AMD3100 and CXCL12 treatments during PBMCs infection with ncp-2 96b2222 BVDV to assess whether changes in concentration might uncover a role for CXCR4 in viral entry; 3) examination of *CXCR4* and *CXCL12*

mRNA expression in maternal and fetal *in vivo* tissues from animals infected with ncp-2 96b2222 BVDV to evaluate the applicability of an *in vitro* model for these studies.

In conclusion, *in vitro* infection of bovine PBMCs with ncp-2 96b2222 BVDV might serve as a useful model for the PI innate immune response and CXCR4 up-regulation. We have shown that PBMCs are able to mount an IFN-I response and have an upregulation of CXCR4 when infected with ncp-2 96b2222 BVDV *in vitro,* as we have shown in PI fetal tissue *in vivo*. Additional experiments designed to identify and explore the association between these molecules may be useful in the study of BVDV persistent infection and may assist in the development of additional *in vivo* investigation.

## LITERATURE CITED


Aiello, S.E. 1998. The Merck Veterinary Manual (Whitehouse Station, Merck and Co.).

Amara, A., Gall, S.L., Schwartz, O., Salamero, J., Montes, M., Loetscher, P., Baggiolini, M., Virelizier, J.L., Arenzana-Seisdedos, F., 1997, HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J Exp Med 186, 139-146.

Audige, A., Urosevic, M., Schlaepfer, E., Walker, R., Powell, D., Hallenberger, S., Joller, H., Simon, H.U., Dummer, R., Speck, R.F., 2006, Anti-HIV state but not apoptosis depends on IFN signature in CD4+ T cells. J Immunol 177, 6227-6237.

Baigent, S.J., Zhang, G., Fray, M.D., Flick-Smith, H., Goodbourn, S., McCauley, J.W., 2002, Inhibition of beta interferon transcription by noncytopathogenic bovine viral diarrhea virus is through an interferon regulatory factor 3-dependent mechanism. J Virol 76, 8979-8988.

Beer, M., Wolf, G., Pichler, J., Wolfmeyer, A., Kaaden, O.R., 1997, Cytotoxic T-lymphocyte responses in cattle infected with bovine viral diarrhea virus. Vet Microbiol 58, 9-22.

Bielefeldt-Ohmann, H., 1995, The pathologies of bovine viral diarrhea virus infection. A window on the pathogenesis. Vet Clin North Am Food Anim Pract 11, 447-476.

Biolchini, A., Curreli, S., Zicchedda, M., Serra, C., Dolei, A., 2000, Opposite effects of IFN-gamma on CCR5 and CXCR4 expression and on entry of M- and T-tropic HIV in epithelial cells. AIDS 14, 613-615.

Brackenbury, L.S., Carr, B.V., Stamataki, Z., Prentice, H., Lefevre, E.A., Howard, C.J., Charleston, B., 2005, Identification of a cell population that produces alpha/beta interferon in vitro and in vivo in response to noncytopathic bovine viral diarrhea virus. J Virol 79, 7738-7744.

Brewoo, J.N., Haase, C.J., Sharp, P., Schultz, R.D., 2007, Leukocyte profile of cattle persistently infected with bovine viral diarrhea virus. Vet Immunol Immunopathol 115, 369-374.

Brownlie, J., Clarke, M.C., Howard, C.J., 1984, Experimental production of fatal mucosal disease in cattle. Vet Rec 114, 535-536.

Brownlie, J., Clarke, M.C., Howard, C.J., 1989, Experimental infection of cattle in early pregnancy with a cytopathic strain of bovine virus diarrhoea virus. Res Vet Sci 46, 307-311.

Brownlie, J., Hooper, L.B., Thompson, I., Collins, M.E., 1998, Maternal recognition of foetal infection with bovine virus diarrhoea virus (BVDV)--the bovine pestivirus. Clin Diagn Virol 10, 141-150.

Burger, J.A., Burkle, A., 2007, The CXCR4 chemokine receptor in acute and chronic leukaemia: a marrow homing receptor and potential therapeutic target. Br J Haematol 137, 288-296.

Burger, J.A., Peled, A., 2009, CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. Leukemia 23, 43-52.

Busillo, J.M., Benovic, J.L., 2007, Regulation of CXCR4 signaling. Biochim Biophys Acta 1768, 952-963.

Charleston, B., Fray, M.D., Baigent, S., Carr, B.V., Morrison, W.I., 2001, Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type I interferon. J Gen Virol 82, 1893-1897.

Chen, Z., Rijnbrand, R., Jangra, R.K., Devaraj, S.G., Qu, L., Ma, Y., Lemon, S.M., Li, K., 2007, Ubiquitination and proteasomal degradation of interferon regulatory factor-3 induced by Npro from a cytopathic bovine viral diarrhea virus. Virology 366, 277-292.

Childs, T., 1946, X Disease of Cattle - Saskatchewan. Can J Comp Med Vet Sci 10, 316-319.

Collen, T., Morrison, W.I., 2000, CD4(+) T-cell responses to bovine viral diarrhoea virus in cattle. Virus Res 67, 67-80.

Confer, A.W., Fulton, R.W., Step, D.L., Johnson, B.J., Ridpath, J.F., 2005, Viral antigen distribution in the respiratory tract of cattle persistently infected with bovine viral diarrhea virus subtype 2a. Vet Pathol 42, 192-199.

Constable, P.D., Hull, B.L., Wicks, J.R., Myer, W., 1993, Femoral and tibial fractures in a newborn calf after transplacental infection with bovine viral diarrhoea virus. Vet Rec 132, 383-385.

Coria, M.F., McClurkin, A.W., 1978, Specific immune tolerance in an apparently healthy bull persistently infected with bovine viral diarrhea virus. J Am Vet Med Assoc 172, 449-451.

Crump, M.P., Gong, J.H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J.L., Baggiolini, M., Sykes, B.D., Clark-Lewis, I., 1997, Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. EMBO J 16, 6996-7007.

Desport, M., Collins, M.E., Brownlie, J., 1998, Genome instability in BVDV: an examination of the sequence and structural influences on RNA recombination. Virology 246, 352-361.

Di Salvo, J., Koch, G.E., Johnson, K.E., Blake, A.D., Daugherty, B.L., DeMartino, J.A., Sirotina-Meisher, A., Liu, Y., Springer, M.S., Cascieri, M.A., Sullivan, K.A., 2000, The CXCR4 agonist ligand stromal derived factor-1 maintains high affinity for receptors in both Galpha(i)-coupled and uncoupled states. Eur J Pharmacol 409, 143-154.

Dolei, A., Biolchini, A., Serra, C., Curreli, S., Gomes, E., Dianzani, F., 1998, Increased replication of T-cell-tropic HIV strains and CXC-chemokine receptor-4 induction in T cells treated with macrophage inflammatory protein (MIP)-1alpha, MIP-1beta and RANTES beta-chemokines. AIDS 12, 183-190.

Dolei, A., Serra, C., Arca, M.V., Tilocca, F., Pietravalle, M., Alemanno, L., Toniolo, A., Ameglio, F., 1994, Mutual interactions between HIV-1 and cytokines in adherent cells during acute infection. Arch Virol 134, 157-168.

Fredriksen, B., Sandvik, T., Loken, T., Odegaard, S.A., 1999, Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Vet Rec 144, 111-114.

Fulton, R.W., Briggs, R.E., Ridpath, J.F., Saliki, J.T., Confer, A.W., Payton, M.E., Duff, G.C., Step, D.L., Walker, D.A., 2005, Transmission of bovine viral diarrhea virus 1b to susceptible and vaccinated calves by exposure to persistently infected calves. Can J Vet Res 69, 161-169.

Gil, L.H., Ansari, I.H., Vassilev, V., Liang, D., Lai, V.C., Zhong, W., Hong, Z., Dubovi, E.J., Donis, R.O., 2006a, The amino-terminal domain of bovine viral diarrhea virus Npro protein is necessary for alpha/beta interferon antagonism. J Virol 80, 900-911.

Gil, L.H., van Olphen, A.L., Mittal, S.K., Donis, R.O., 2006b, Modulation of PKR activity in cells infected by bovine viral diarrhea virus. Virus Res 116, 69-77.

Goens, S.D., 2002, The evolution of bovine viral diarrhea: a review. Can Vet J 43, 946-954.

Goyal, R.J.F.S.M. 2005. Bovine Viral Diarrhea Virus, Goyal, R.J.F.S.M., ed. (Ames, IA, Blackwell Publishing), p. 261.

Hamers, C., Dehan, P., Couvreur, B., Letellier, C., Kerkhofs, P., Pastoret, P.P., 2001, Diversity among bovine pestiviruses. Vet J 161, 112-122.

Hatse, S., Balzarini, J., Liekens, S., 2006, Stromal cell-derived factor 1 (CXCL12) binds to endothelial cells and signals through a receptor different from CXCR4. Biochem Biophys Res Commun 348, 192-199.

Houe, H., 2003, Economic impact of BVDV infection in dairies. Biologicals 31, 137-143.

Howard, C.J., Clarke, M.C., Sopp, P., Brownlie, J., 1992, Immunity to bovine virus diarrhoea virus in calves: the role of different T-cell subpopulations analysed by specific depletion in vivo with monoclonal antibodies. Vet Immunol Immunopathol 32, 303-314.

Janeway CA Jr, T.P., Walport M, Shlomchik MJ, 2005, Immunobiology: the immune system in health and disease, 6th Edition. Garland Science Publishing, New York City.

Johnson, D.W., Muscoplat, C.C., 1973, Immunologic abnormalities in calves with chronic bovine viral diarrhea. Am J Vet Res 34, 1139-1141.

Jones, R., Nelson, M., 2007, The role of receptors in the HIV-1 entry process. Eur J Med Res 12, 391-396.

Jubb, K.V.P., Kennedy, P.C., 1963, Pathology of Domestic Animals, Vol 2, 1st Edition. New York Academic Press.

Kirkland, P.D., Richards, S.G., Rothwell, J.T., Stanley, D.F., 1991, Replication of bovine viral diarrhoea virus in the bovine reproductive tract and excretion of virus in semen during acute and chronic infections. Vet Rec 128, 587-590.

Kumar, A., Humphreys, T.D., Kremer, K.N., Bramati, P.S., Bradfield, L., Edgar, C.E., Hedin, K.E., 2006, CXCR4 physically associates with the T cell receptor to signal in T cells. Immunity 25, 213-224.

Kummerer, B.M., Tautz, N., Becher, P., Thiel, H., Meyers, G., 2000, The genetic basis for cytopathogenicity of pestiviruses. Vet Microbiol 77, 117-128.

Lecot, S., Belouzard, S., Dubuisson, J., Rouille, Y., 2005, Bovine viral diarrhea virus entry is dependent on clathrin-mediated endocytosis. J Virol 79, 10826-10829.

Lee, K.M., Gillespie, J.H., 1957, Propagation of virus diarrhea virus of cattle in tissue culture. Am J Vet Res 18, 952-953.

Liu, K.K., Dorovini-Zis, K., 2009, Regulation of CXCL12 and CXCR4 expression by human brain endothelial cells and their role in CD4+ and CD8+ T cell adhesion and transendothelial migration. J Neuroimmunol 215, 49-64.

Loo, Y.M., Owen, D.M., Li, K., Erickson, A.K., Johnson, C.L., Fish, P.M., Carney, D.S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., Lee, W.M., Hagedorn, C.H., Lau, D.T., Weinman, S.A., Lemon, S.M., Gale, M., Jr., 2006, Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci U S A 103, 6001-6006.

Maekawa, T., Ishii, T., 2000, Chemokine/receptor dynamics in the regulation of hematopoiesis. Intern Med 39, 90-100.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984, Production of cattle immunotolerant to bovine viral diarrhea virus. Can J Comp Med 48, 156-161.

Moriuchi, H., Moriuchi, M., Fauci, A.S., 1997, Cloning and analysis of the promoter region of CCR5, a coreceptor for HIV-1 entry. J Immunol 159, 5441-5449.

Moriuchi, M., Moriuchi, H., Margolis, D.M., Fauci, A.S., 1999, USF/c-Myc enhances, while Yin-Yang 1 suppresses, the promoter activity of CXCR4, a coreceptor for HIV-1 entry. J Immunol 162, 5986-5992.

Nagasawa, T., Tachibana, K., Kishimoto, T., 1998, A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin Immunol 10, 179-185.

Neel, N.F., Schutyser, E., Sai, J., Fan, G.H., Richmond, A., 2005, Chemokine receptor internalization and intracellular trafficking. Cytokine Growth Factor Rev 16, 637-658.

Ohmann, H.B., 1982, Experimental fetal infection with bovine viral diarrhea virus. II. Morphological reactions and distribution of viral antigen. Can J Comp Med 46, 363-369.

Ohmann, H.B., Lawman, M.J., Babiuk, L.A., 1987, Bovine interferon: its biology and application in veterinary medicine. Antiviral Res 7, 187-210.

Olafson P, M.A., Fox a, 1946, An apparently new transmissible disease of cattle. Cornell Vet 36, 205-213.

Paton, D.J., Goodey, R., Brockman, S., Wood, L., 1989, Evaluation of the quality and virological status of semen from bulls acutely infected with BVDV. Vet Rec 124, 63-64.

Payne, D., Drinkwater, S., Baretto, R., Duddridge, M., Browning, M.J., 2009, Expression of chemokine receptors CXCR4, CXCR5 and CCR7 on B and T lymphocytes from patients with primary antibody deficiency. Clin Exp Immunol 156, 254-262.

Radostits OM, L.I., 1988, New Concepts in the pathogenesis, diagnosis, and control of disease caused by the bovine viral diarrhea virus. Can Vet J 29, 513-528.

Rhodes, S.G., Cocksedge, J.M., Collins, R.A., Morrison, W.I., 1999, Differential cytokine responses of CD4+ and CD8+ T cells in response to bovine viral diarrhoea virus in cattle. J Gen Virol 80 ( Pt 7), 1673-1679.

Ridpath, J.F., 2003, BVDV genotypes and biotypes: practical implications for diagnosis and control. Biologicals 31, 127-131.

Ridpath, J.F., Bolin, S.R., Dubovi, E.J., 1994, Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66-74.

Rinaldo, C.R., Jr., Isackson, D.W., Overall, J.C., Jr., Glasgow, L.A., Brown, T.T., Bistner, S.I., Gillespie, J.H., Scott, F.W., 1976, Fetal and adult bovine interferon production during bovine viral diarrhea virus infection. Infect Immun 14, 660-666.

Ronecker, S., Zimmer, G., Herrler, G., Greiser-Wilke, I., Grummer, B., 2008, Formation of bovine viral diarrhea virus E1-E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains. J Gen Virol 89, 2114-2121.

Schweizer, M., Matzener, P., Pfaffen, G., Stalder, H., Peterhans, E., 2006, "Self" and "nonself" manipulation of interferon defense during persistent infection: bovine viral diarrhea virus resists alpha/beta interferon without blocking antiviral activity against unrelated viruses replicating in its host cells. J Virol 80, 6926-6935.

Schweizer, M., Peterhans, E., 2001, Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J Virol 75, 4692-4698.

Sentsui, H., Murakami, K., Inoshima, Y., Yokoyama, T., Inumaru, S., 2001, Anti-viral effect of recombinant bovine interferon gamma on bovine leukaemia virus. Cytokine 16, 227-231.

Serra, C., Biolchini, A., Mei, A., Kotenko, S., Dolei, A., 2008, Type III and I interferons increase HIV uptake and replication in human cells that overexpress CD4, CCR5, and CXCR4. AIDS Res Hum Retroviruses 24, 173-180.

Shoemaker, M.L., Smirnova, N.P., Bielefeldt-Ohmann, H., Austin, K.J., van Olphen, A., Clapper, J.A., Hansen, T.R., 2009, Differential expression of the type I interferon pathway during persistent and transient bovine viral diarrhea virus infection. J Interferon Cytokine Res 29, 23-35.

Shoemaker, M.L., Smirnova, N.P., Bielefeldt-Ohmann, H., Austin, K.J., Van Olphen, A.L., Clapper, J.A., Hansen, T.R., 2008, Differential Expression of the Type I Interferon Pathway during Persistent and Transient Bovine Viral Diarrhea Virus Infection. Journal of Interferon and Cytokine Research 29, 23-26.

Smirnova, N., Troyer, J.L., Schissler, J., Terwee, J., Poss, M., VandeWoude, S., 2005, Feline lentiviruses demonstrate differences in receptor repertoire and envelope structural elements. Virology 342, 60-76.

Smirnova, N.P., Bielefeldt-Ohmann, H., Van Campen, H., Austin, K.J., Han, H., Montgomery, D.L., Shoemaker, M.L., van Olphen, A.L., Hansen, T.R., 2008, Acute non-cytopathic bovine viral diarrhea virus infection induces pronounced type I interferon response in pregnant cows and fetuses. Virus Res 132, 49-58.

Smirnova, N.P., Ptitsyn, A.A., Austin, K.J., Bielefeldt-Ohmann, H., Van Campen, H., Han, H., van Olphen, A.L., Hansen, T.R., 2009, Persistent fetal infection with bovine viral diarrhea virus differentially affects maternal blood cell signal transduction pathways. Physiol Genomics 36, 129-139.

Sodhi, A., Montaner, S., Gutkind, J.S., 2004, Viral hijacking of G-protein-coupled-receptor signalling networks. Nat Rev Mol Cell Biol 5, 998-1012.

Stokstad, M., Loken, T., 2002, Pestivirus in cattle: experimentally induced persistent infection in calves. J Vet Med B Infect Dis Vet Public Health 49, 494-501.

Swasdipan, S., McGowan, M., Phillips, N., Bielefeldt-Ohmann, H., 2002, Pathogenesis of transplacental virus infection: pestivirus replication in the placenta and fetus following respiratory infection. Microb Pathog 32, 49-60.

Underdahl, N.R., Grace, O.D., Hoerlein, A.B., 1957, Cultivation in tissue-culture of cytopathogenic agent from bovine mucosal disease. Proc Soc Exp Biol Med 94, 795-797.

Uttenthal, A., Hoyer, M.J., Grondahl, C., Houe, H., van Maanen, C., Rasmussen, T.B., Larsen, L.E., 2006, Vertical transmission of bovine viral diarrhoea virus (BVDV) in mousedeer (Tragulus javanicus) and spread to domestic cattle. Arch Virol 151, 2377-2387.

Vila-Coro, A.J., Rodriguez-Frade, J.M., Martin De Ana, A., Moreno-Ortiz, M.C., Martinez, A.C., Mellado, M., 1999, The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. FASEB J 13, 1699-1710.

Virelizier, J.L., 1999, Blocking HIV co-receptors by chemokines. Dev Biol Stand 97, 105-109.

Volin, M.V., Joseph, L., Shockley, M.S., Davies, P.F., 1998, Chemokine receptor CXCR4 expression in endothelium. Biochem Biophys Res Commun 242, 46-53.

Wegner, S.A., Ehrenberg, P.K., Chang, G., Dayhoff, D.E., Sleeker, A.L., Michael, N.L., 1998, Genomic organization and functional characterization of the chemokine receptor CXCR4, a major entry co-receptor for human immunodeficiency virus type 1. J Biol Chem 273, 4754-4760.

Wilkins, C., Gale, M., Jr., Recognition of viruses by cytoplasmic sensors. Curr Opin Immunol.

Zhang, J.C., Sun, L., Nie, Q.H., Huang, C.X., Jia, Z.S., Wang, J.P., Lian, J.Q., Li, X.H., Wang, P.Z., Zhang, Y., Zhuang, Y., Sun, Y.T., Bai, X., 2009, Down-regulation of CXCR4 expression by SDF-KDEL in CD34(+) hematopoietic stem cells: An anti-human immunodeficiency virus strategy. J Virol Methods 161, 30-37.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., Littman, D.R., 1998, Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393, 595-599.